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13. ABSTRACT (Maximum 200 Words)

Homeobox genes represent a class of transcription factors important in embryogenesis, organogenesis, cell growth and differentiation, and cell migration. However, there is little known about their role in regulating endothelial cell (EC) phenotype in response to proangiogenic factors secreted by breast cancer, although at least two homeobox genes have been implicated in inducing the angiogenic phenotype in ECs. We are therefore testing the homeobox gene *Gax* regulates breast cancer-induced angiogenesis through its ability to regulate the expression of downstream target genes in ECs. Using a quantitative real-time PCR assay and *in situ* hybridization, we have found that *Gax* expression inhibits *in vitro* angiogenesis. Moreover, by cDNA microarray, we have found that *Gax* expression is downregulated by proangiogenic factors and that *Gax* downregulates pro-angiogenic adhesion molecules in ECs and upregulates the cyclin-dependent kinase inhibitor p19INK4D. These observations will allow us to study the mechanism of *Gax*-mediated activation or repression of their expression to be studied and will form the basis for future studies that will examine in more detail the mechanism by which *Gax* activates downstream target genes and the detailed signaling pathways involved in this activation. Given the profound effect *Gax* has on endothelial cell activation, it is likely that these studies will identify new molecular targets for the antiangiogenic therapy of breast cancer. Ultimately, these same techniques will be applied to other homeobox genes implicated in regulating EC phenotype during breast cancer-induced angiogenesis.

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INTRODUCTION

Homeobox genes represent a class of transcription factors important in embryogenesis, organogenesis, cell growth and differentiation, and cell migration (1-6). However, there is little known about their role in regulating endothelial cell (EC) phenotype in response to pro- and antiangiogenic factors secreted by breast cancer cells and the surrounding normal stroma. When we originally submitted our proposal, only two homeobox genes, *HOXD3* and *HOXB3*, had been implicated in regulating tumor-induced angiogenesis (2, 7, 8). Since then, two more (*HOXD10* and *Hex*) have been implicated by others (9-11), and we have developed more evidence implicating another (*Gax*) (12). Because, of the handful of homeobox genes implicated in regulating angiogenesis, only *Gax* shows a strong restriction in its expression to cardiovascular tissues in the adult (13, 14), we originally proposed to test the hypothesis that *Gax* (13-29) regulates breast cancer-induced angiogenesis through its ability to regulate the expression of specific downstream target genes in vascular endothelial cells (ECs) We based this hypothesis on our preliminary data showing that *Gax* is expressed in vascular ECs (18) and inhibits EC proliferation *in vitro* (18). Using a quantitative real-time PCR assay and *in situ* hybridization, we planned to examine the effect of breast cancer-secreted proangiogenic peptides and and antiangiogenic therapies on *Gax* expression. Next, using an adenovirus expressing *Gax* (30), we proposed to drive *Gax* expression in ECs in order to determine the effect of *Gax* expression on breast cancer angiogenesis, both *in vitro* and *in vivo*. Finally, because few downstream targets of *Gax* have been identified (23, 28, 30), we proposed to evaluate the changes in global gene expression in ECs that result from *Gax* expression. These observations will help us to identify likely downstream targets of *Gax*, allowing the mechanism of *Gax*-mediated activation or repression of their expression to be studied. Our results will form the basis for future studies that will examine in more detail the mechanism by which *Gax* activates downstream target genes and the detailed signaling pathways involved in this activation. Given the profound effect *Gax* has on endothelial cell activation, it is likely that these studies will identify new molecular targets for the antiangiogenic therapy of breast cancer. Ultimately, these same techniques will be applied to other homeobox genes implicated in regulating EC phenotype during breast cancer angiogenesis.

BODY

Background

In order to grow and metastasize, breast malignancies are critically dependent upon inducing the ingrowth of blood vessels from the host (31, 32). Numerous studies have suggested a correlation between secretion of proangiogenic molecules and increased angiogenesis and increased likelihood of lymph node metastases with poorer prognosis in breast cancer (33, 34). Inhibition of tumor-induced angiogenesis has thus emerged in the last decade as a promising new strategy for breast cancer therapy, either alone or in combination with conventional therapies (35-38). During angiogenesis, whether physiologic or tumor-induced, vascular ECs undergo distinct changes in phenotype and gene expression, including activation of proteolytic enzymes to degrade basement membrane, sprouting, proliferation, tube formation, and production of extracellular matrix (39-41). Although the EC receptors and signaling pathways activated by proangiogenic factors secreted by breast cancer cells, such as vascular endothelial growth factor (VEGF) (42, 43) and basic fibroblast growth factor (bFGF) (42), have been extensively studied (44-46), much less is known about the molecular biology of the downstream transcription factors

activated by these signaling pathways, which then activate the genes necessary for EC phenotypic changes during breast cancer-induced angiogenesis.

Homeobox genes encode transcription factors containing a common DNA-binding motif (1, 4-6, 47). Important regulators of body plan and cell fate during embryogenesis, homeobox genes also have pleiotropic roles in many cell types in the adult and can modulate cell cycle progression and arrest, cell differentiation, migration, and apoptosis (1, 3-5, 7, 12, 48, 49). As a gene family, they are thus excellent candidates to be involved in the final transcriptional control of genes responsible for the changes in EC phenotype induced by breast cancer-secreted proangiogenic factors. Until recently, little was known about how homeobox genes might influence angiogenesis. There is now evidence for their involvement in phenotypic changes ECs undergo during angiogenesis (7-9, 11, 12). For instance, one homeobox gene, *HOXD3*, induces the expression of $\alpha_V\beta_3$, an integrin important in angiogenesis (50), resulting in the conversion of ECs to an angiogenic phenotype both *in vitro* and *in vivo* (7); impaired *HOXD3* expression is associated with impaired angiogenesis in a mouse model (49). Similarly, overexpression of the homeobox gene *HOXB3* results in an increase in capillary vascular density and angiogenesis (8). Taken together, these data suggest significant roles for specific homeobox genes in responding to extracellular signals and activating downstream genes to induce phenotypic changes associated with breast cancer-induced angiogenesis. Since the submission of our original proposal, two additional homeobox genes have been implicated in the regulation of EC phenotype during angiogenesis. In contrast to *HOXB3* and *HOXD3*, another HOX cluster gene, *HOXD10*, inhibits EC conversion to the angiogenic phenotype (9). *HOXD10* expression is higher in quiescent endothelium than in tumor-associated vascular endothelium. In addition, sustained expression of *HOXD10* inhibits EC migration and blocks bFGF- and VEGF-induced angiogenesis in the chick chorioallantoic membrane assay *in vivo*. Consistent with these observations, human ECs overexpressing *HOXD10* fail to form new blood vessels (9) when embedded in Matrigel-containing sponges (51) in nude mice. Similarly, *Hex* overexpression in human umbilical vein endothelial cells (HUVECs) inhibits *in vitro* surrogates for angiogenesis, including migration towards vascular endothelial growth factor (VEGF), invasion, proliferation, and tube formation on reconstituted basement membrane (Matrigel) (11). Given that previous data showing high levels of *Hex* expression in proliferating vasculature had suggested that *Hex* would be more likely to induce EC proliferation and angiogenesis (52, 53), the observation that *Hex* inhibits *in vitro* angiogenesis suggests a more complex role for this gene than previously understood.

The cardiovascular-specific homeobox gene *Gax* appears more likely to function as a negative regulator of angiogenesis in ECs, like *HOXD10*. After isolating it from a rat aorta cDNA library (13, 54), we showed that *Gax* has profound effects on cardiovascular tissues (30, 55). In vascular smooth muscle cells (VSMCs) *Gax* expression is downregulated by mitogenic signals and upregulated by growth arrest signals (13, 29). Consistent with this observation, *Gax* induces G_1 cell cycle arrest (30) and can induce apoptosis in VSMCs under stress (22). Also, *Gax* overexpression inhibits VSMC migration, downregulating the expression of integrins, $\alpha_V\beta_3$ and $\alpha_V\beta_5$, both of which are associated with the activated ("synthetic") state in VSMCs and the angiogenic phenotype in ECs (28, 50). *In vivo*, *Gax* expression in arteries inhibits proliferative restenosis of the arterial lumen after injury (19, 20, 23, 30). We now have evidence that *Gax* mRNA is also expressed in ECs (12). Understanding the actions of *Gax* on downstream target genes, as well as signals that activate or repress *Gax* expression, could thus lead to a better

understanding of the mechanisms of breast cancer-induced angiogenesis and the identification of new molecular targets for the antiangiogenic therapy of breast cancer.

Little is known about specific transcription factors involved in the control of endothelial cell phenotype during breast cancer-induced angiogenesis, especially transcription factors that inhibit angiogenesis. *Gax* is a novel homeobox transcription factor (13) whose antiproliferative (28), pro-apoptotic (22), and antimigratory (28) activities were originally characterized by us and others in vascular smooth muscle. Although other homeobox genes have been implicated in promoting or inhibiting EC conversion to the angiogenic phenotype (7-9, 11, 12), *Gax* is the only homeobox transcription factor described thus far whose expression is largely restricted to the cardiovascular system in the adult (13, 14). It is thus a new and unique candidate transcription factor for regulating EC conversion to the angiogenic phenotype in response to proangiogenic factors secreted by breast tumors. The hypothesis that *Gax* inhibits the phenotypic changes in ECs that occur when they are stimulated by the proangiogenic factors secreted by breast cancer cells represents an innovative approach to the study of the transcriptional control of EC phenotypic changes during angiogenesis, as does our use of *Gax* as a molecular tool to study the mechanisms of breast cancer-induced angiogenesis. More importantly, identification of downstream targets of *Gax* could identify previously unsuspected molecular targets for the antiangiogenic therapy of breast cancer and other tumors, leading to new lines of investigation into breast cancer-induced angiogenesis and new therapies based on these observations. Thus, the studies we have proposed and undertaken with support from the Department of Defense have attempted to use *Gax* as a molecular tool to: (1) enhance our understanding of the mechanisms by breast cancer stimulates endothelial cells to become angiogenic; and (2) provide the basis for the design of antiangiogenic therapies of breast cancer targeting *Gax* or its downstream targets.

Overview of progress over the last year

Since this project began, we have made considerable progress in meeting the milestones originally proposed in our original Statement of Work. We have accomplished on time all but one of the tasks originally proposed for Year One and are ahead of schedule on others. For most of the remaining tasks, we are on or close to being on schedule. We have accomplished this in spite of several difficulties. First, Dr. Edward Owuor was unable to begin work in the lab until the end of Month 3 (July 1, 2002). Then, before he was fully trained, Dr. Owuor abruptly resigned without giving notice in August 2002 and accepted a position at the Forensics Laboratory at the FAA Civil Aerospace Medical Institute in Oklahoma City. Because we were not able to find a qualified replacement for Dr. Ouwor until late April 2003, when Sejal Patel, Ph.D., began work in our laboratory, for nearly all of Year One we were without a Ph.D.-level laboratory supervisor. Fortunately, Dr. Patel is rapidly learning our laboratory's techniques and assays, and we anticipate that she will quickly make an impact to help us get back on schedule for the tasks in the Statement of Work in which we have fallen behind. The second major difficulty to be overcome is that the principal investigator was unable to work or provide supervision for his laboratory during the greater part of October 2002, due to serious medical problems of a family member. Given these difficulties, we believe we have still managed to make excellent progress and should still be able to achieve the majority of milestones proposed in Year Two. It is possible that during Year Two we will need to make adjustments to the overall plan, and we will contact the appropriate personnel to do so, should it become necessary.

Detailed progress report by tasks in the original Statement of Work

Task 1: ***Characterize the regulation of Gax expression in three different endothelial cell types in vitro, months 1 to 24:***

a. Develop and verify real time quantitative reverse transcriptase polymerase chain reaction assay to measure *Gax* transcript levels. (Months 1-6.)

Status: Accomplished.

Results and Discussion: We have successfully developed a quantitative real time PCR assay to measure *Gax* mRNA levels on schedule. Initially, we used SYBR Green as our detection method in preliminary experiments. However, melting curves performed using a variety of *Gax*-specific primer sets demonstrated that primer-dimer is frequently present at levels that severely interfere with interpretation of data, especially given the limitations of the software suite that came with our equipment (data not shown). Because *Gax* message is of low abundance, the presence of primer-dimer can potentially compromise our accuracy. Therefore, we began to utilize TaqMan probes (56, 57). We generally used carboxyfluorescein fluorescent dye 6-FAM as the 5'-fluorophore and Black Hole Quencher-1 (BHQ-1, Biosearch Technologies, Inc.) as the 3'-quencher.

Primer and probe design. For our proposed experiments involving real time quantitative PCR, we used the MacVector v.7.1 DNA analysis software package to design specific primers and TaqMan probes. The primer/probe set that we are currently using to measure *Gax* transcript amplifies a 238 bp sequence of the human *Gax* coding sequence (58) between bases 803 and 1040. The probe binds to bases 962 to 982 and has a calculated melting point of 69.5° under the reaction conditions used. We normalize to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using primers that amplify 138 bp fragment from 572 to 709 of the human gene and a probe that binds from 625 to 644 (59). Before the primers and probes were synthesized, their sequences were subjected to a BLAST (60, 61) search against the Genbank database, in order to detect any possibility that they might bind to or amplify genes other than the ones for which they were designed. Further, all reactions were subjected to agarose gel electrophoresis, to verify that the PCR reaction products were of the correct size.

RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR). Before running assays on experimental samples, each primer/probe set, annealing conditions, Mg^{2+} concentration, and primer and probe concentration were optimized using plasmids containing the *Gax* cDNA (13). We were able to detect as few as 10-100 copies of the *Gax* cDNA in our assays. Because the *Gax* gene has a single exon (58), all RNA samples were treated with RNA-free DNAse prior to reverse transcription, and random RNA samples are subjected to sham reverse transcription (no reverse transcriptase) and real time PCR with the GAPDH primer/probe set, to verify that there was no genomic DNA contamination. The PCR cycle consisted of an initial 2.5 minute denaturation step at 95° C, followed by 40 cycles of denaturation at 95° C for 15 seconds, annealing at the appropriate annealing temperature for each primer for 15 to 30 seconds, and extension at 72° C for 15 to30 seconds, with exact conditions depending upon the specific probe/primer set.

Normalization and quantification. In our preliminary experiments, we estimated relative *Gax* levels by calculating the differences in threshold cycle (C_t) between *Gax* and our control gene ($\Delta Ct = C_t^{GAPDH} - C_t^{Gax}$) and used the formula: *Gax* level $\approx 2^{-\Delta Ct}$. (For results of these

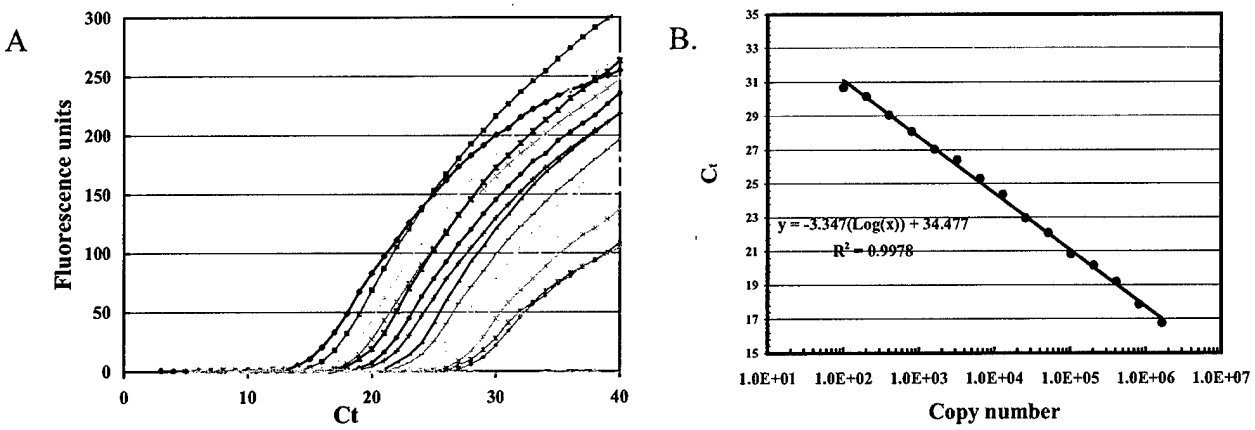

Figure 1. Representative real time PCR standard curve for *Gax*. Using primers and a TaqMan probe (5' end=FAM, 3' end=BHQ1) specific for *Gax*, serial dilutions of the Gax cDNA from 1.64 million to 100 copies were subjected to real time quantitative PCR and a standard curve produced. There was an excellent linear fit to the semilog curve (r^2=0.998). The calculated amplification efficiency from the standard curve slope was 98.9%.

experiments, see Task 1b, below.) While this method is useful for estimating relative levels of a gene and changes in expression, it depends upon the assumption that the PCR efficiency is identical for the *Gax* and GAPDH primer sets (56, 62). While this is approximately true for *Gax* and GAPDH (data not shown), it may not be true for primer sets designed for other genes we proposed to examine. For future experiments we have developed a more rigorous method. Using the primers from the TaqMan probe/primer sets, we amplify specific PCR products for RNA samples known to be positive for the gene of interest by conventional PCR. These are then subjected to electrophoresis on agarose gels and the specific PCR product bands cut from the gel and extracted using Qiaex II (Qiagen, Inc.). The fragments were serially diluted in log steps from 10^8 copies to 10 copies in a 1 μl volume and amplified in real time PCR reactions. Calibration curves were then constructed by making a semilog plot of C_t versus the known copy number for each plasmid. In Figure 1, we present a representative real time PCR experiment, in which a standard curve for *Gax* has been constructed by two-fold serial dilutions of the full length *Gax* cDNA.

Plan for Year Two: We have used our real time quantitative PCR assay to measure *Gax* transcript levels in HUVECs and HMEC-1 cells in response to mitogens and proangiogenic stimuli (see below). We plan to confirm these results and proceed to measure changes in *Gax* mRNA levels in three different endothelial cell types in response to growth factors, pro-angiogenic, and antiangiogenic factors. Further, we will develop real time quantitative PCR assays based on standard curves rather than the $\Delta\Delta C_t$ method (62). Total RNA will be used to determine the copy number for each transcript of interest. Samples will be diluted if necessary to bring their C_t within the linear range of the semilog calibration curve. To correct for differences in RNA quality and quantity between samples, data will normalized by dividing the copy number of the target cDNA by the copy number of GAPDH and results presented as copies of target gene per 10,000 copies of GAPDH.

b. Measure changes in *Gax* mRNA levels in three different endothelial cell types in response to growth factors, pro-angiogenic, and antiangiogenic factors. (Months 6-24)

Status: In progress.

Results and Discussion: Because *Gax* is expressed at a low level even in VSMCs, detection of changes in its message level is difficult using Northern blot or RNAse protection assays, frequently requiring exposure times of several days (13). Consequently, we used a quantitative real time PCR assay with *Gax*-specific primers and a TaqMan probe (57), according to methods described in Task 1a. First, we tested whether serum and the two proangiogenic factors thought to be the most important in regulating tumor angiogenesis affected *Gax* expression in ECs. HMEC-1 cells, a human microvascular endothelial cell line immortalized with the SV40 large T antigen (63), were plated in differing concentrations of serum, bFGF, or VEGF, incubated overnight, and then harvested for RNA isolation and subsequent quantitative real time PCR. *Gax* expression was downregulated by up to ten-fold in cells plated in serum-containing medium (Figure 2A) and by more than two-fold in cells plated in serum-free medium in the presence of VEGF or bFGF (Figure 2B). Similar results were obtained in HUVECs. Next, we studied the time course of *Gax* downregulation by mitogens. HUVECs made quiescent by incubation for 24 hrs in 0.1% FBS were stimulated with 10% FBS plus 5 ng/ml VEGF. *Gax* was rapidly downregulated by 4- to 5-fold within four hours (Figure 3, A and C). Conversely, when randomly cycling HUVECs were placed in medium containing 0.1% serum, *Gax* was upregulated nearly 10-fold within 24 hours (Figure 5B). This regulation and its time course are similar to that observed in quiescent VSMCs stimulated with serum, platelet-derived growth factor, and other mitogens (13, 27, 29, 54). The observation that *Gax* is also expressed in vascular ECs and is regulated in a similar manner suggests that *Gax* expression in ECs is likely to be regulated by other pro- and anti-angiogenic factors, just as *Gax* is regulated in VSMCs by factors that stimulate intimal hyperplasia, such as platelet-derived growth factor and angiotensin II (13, 29).

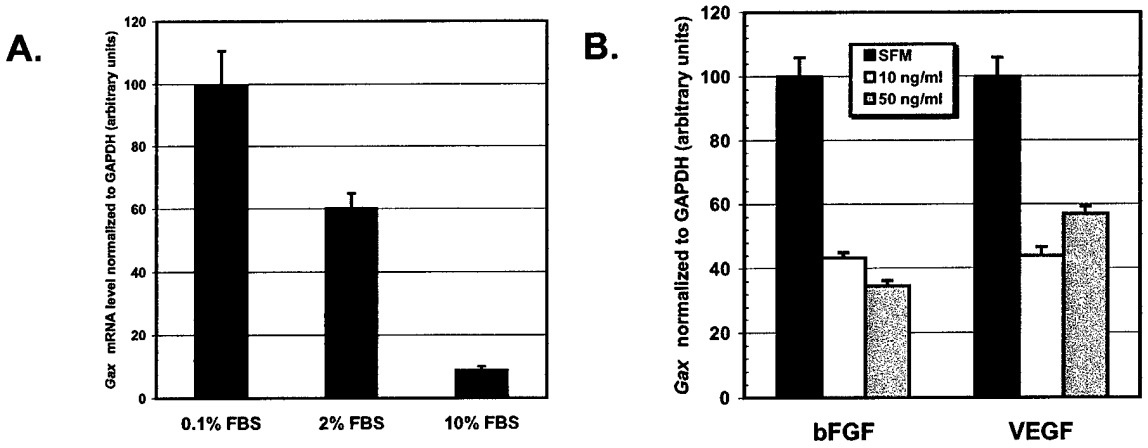

Figure 2. ***Gax expression is downregulated in endothelial cells by serum and proangiogenic factors.*** **A.** Confluent HMEC-1 cells were trypsinized and plated at low density in 100 mm plates in MCDB 131 medium containing the percent serum indicated, incubated overnight, then harvested for RNA isolation and subsequent real time PCR for *Gax* and GAPDH. **B.** HMEC-1 cells were plated in 0.1% serum and allowed to attach for 3 hrs. The media was then replaced with serum-free medium and the indicated concentration of either bFGF or $VEGF_{165}$. The cells were incubated overnight and then harvested for total RNA, which was then subjected to real time PCR for *Gax* and GAPDH. For both experiments, *Gax* levels were normalized to GAPDH and the highest *Gax* level was arbitrarily set at 100. **FBS=fetal bovine serum. SFM= serum free medium.**

Plan for Year Two: We plan to study more carefully the time course of *Gax* regulation in response to proangiogenic factors secreted by breast cancer cells, and to examine whether *Gax* downregulation occurs when ECs are stimulated with other pro-angiogenic factors besides

VEGF and bFGF. Next, ECs will be treated with common antiangiogenic agents, including anti-VEGF antibody, anti-bFGF antibody, angiostatin, endostatin, and TNP-470, and *Gax* expression measured.

c. Measure changes in *Gax* mRNA levels *in vitro* using three different endothelial cell types in response to common cytotoxic therapies used in breast cancer, including chemotherapy and radiation. (Months 6-24.)

Status: In progress.

Results and Discussion: We are in the initial stages of performing clonogenic assays in order to estimate dosages toxic to HUVECs and HMEC-1 cells and to determine the doses that kill 50% of the cells.

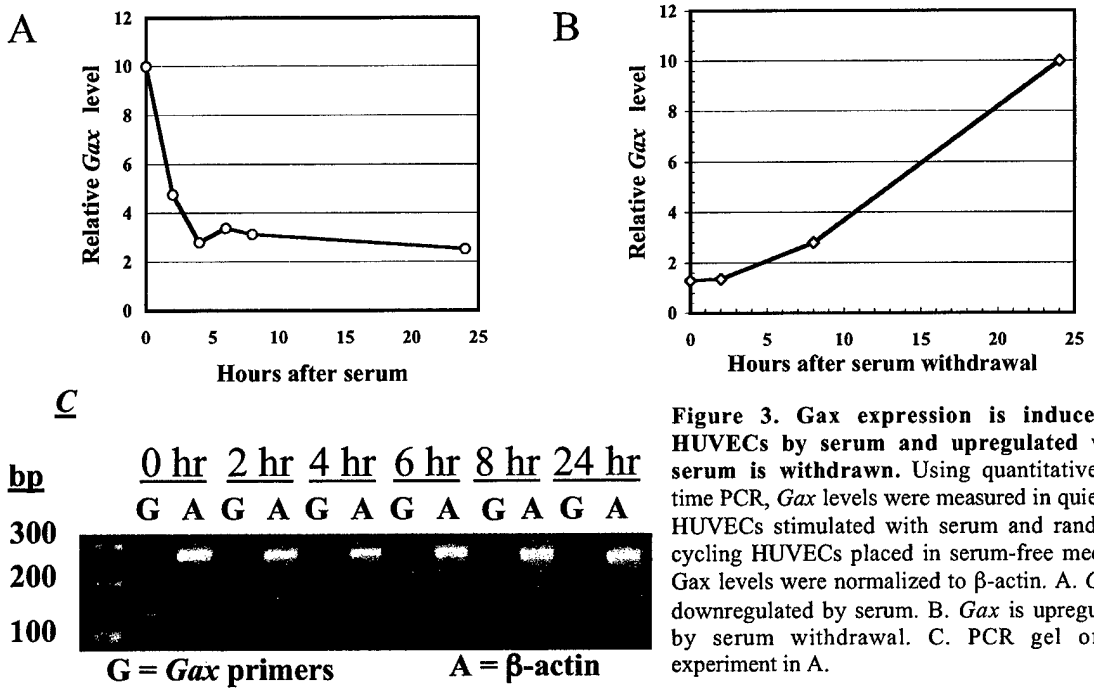

Figure 3. Gax expression is induced in HUVECs by serum and upregulated when serum is withdrawn. Using quantitative real time PCR, *Gax* levels were measured in quiescent HUVECs stimulated with serum and randomly cycling HUVECs placed in serum-free medium. Gax levels were normalized to β-actin. A. *Gax* is downregulated by serum. B. *Gax* is upregulated by serum withdrawal. C. PCR gel of the experiment in A.

Plan for Year Two: The effect of common antineoplastic therapies on expression will be determined, including ionizing radiation according to methods we have used before (36, 38) doxorubicin, 4-hydroxycyclophosphamide (the active metabolite of cyclophosphamide), paclitaxel, methotrexate, and 5-fluorouracil. A variety of doses will be used between zero and the dose that kills 50% of ECs in clonogenic assays. For any positive results obtained, time course and dose-response curves will be generated, and determination of the level of regulation of *Gax* expression made. We anticipate that we should still be able to accomplish these experiments before the end of Year Two

d. Mechanistic studies to determine if regulation of *Gax* expression occurs at the level of transcription, translation, or mRNA stability and mapping of the *Gax* promoter, if necessary. (Months 12-24.)

Status: Not done.

Plan for Year Two: These experiments will be carried out during the Year Two of the project, as proposed in Specific Aim #1 the original Career Development Award.

Task 2:*Measure differences in Gax expression between angiogenic blood vessels and normal blood vessels in vivo. (Months 13 to 36.)*

a. Measure breast cancer cell line-induced angiogenesis *in vivo* using the Matrigel plug assay and breast cancer cell line-conditioned media, and measure *Gax* expression in endothelial cells *in vivo*. This will require 100 mice. (Months 13-24.)

Status: In progress.

Results and Discussion: *In vitro studies*. We are presently collecting conditioned media from the breast cancer cell lines in order to test the ability of the media to induce angiogenesis and to downregulate *Gax* in ECs, as proposed in Specific Aim #1. We anticipate that these in vitro experiments should be complete in the next 3-6 months.

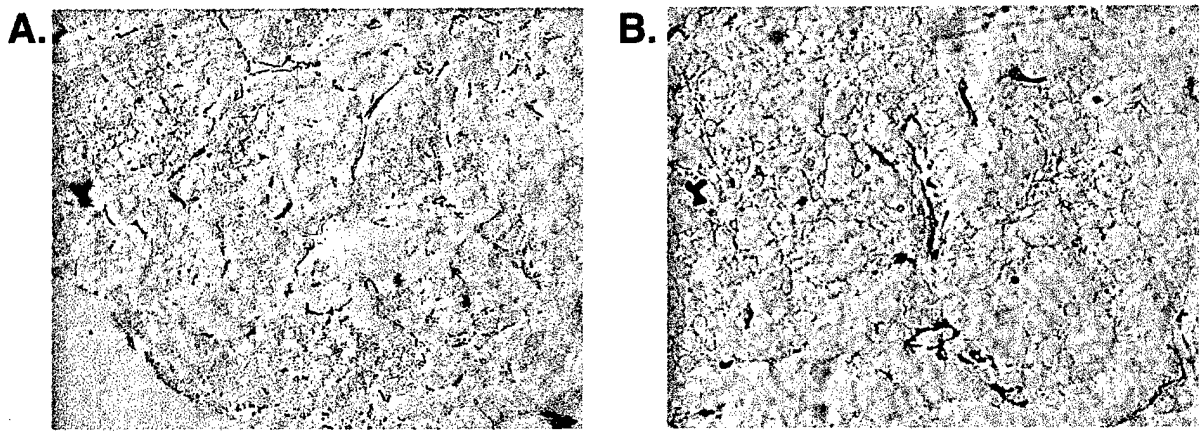

Figure 4. Example of the Matrigel plug assay. Matrigel mixed with A549 cells, which are derived from human lung cancer and secrete numerous pro-angiogenic factors, was implanted into the flanks of athymic nude mice and then harvested two weeks later for frozen sectioning and CD31 staining. A brisk angiogenic response is seen. **A.** Low power. **B.** High power.

In vivo studies. In order to measure *Gax* expression in angiogenic blood vessels, we have begun developing the Matrigel plug assay as described in Specific Aim #2 (64, 65). To begin, we have implanted Matrigel plugs in the flanks of C57BL/6 mice and performed immunohistochemistry with anti-CD31 antibodies in order to identify the neovasculature migrating into the plug. Initially, in pilot experiments we had difficulties visualizing any blood vessels growing into the VEGF- and bFGF-containing Matrigel plugs implanted in C57BL/6 mice, and saw few vessels growing into the plugs (data not shown). For these pilot experiments, we had been fixing our Matrigel plugs in formalin prior to immunohistochemistry. We therefore consulted a colleague, Dr. John Langenfeld (Department of Surgery, UMDNJ-Robert Wood Johnson Medical School), who has been successfully using the Matrigel plug assay in nude mice. He proposed that we would likely obtain more reproducible results if frozen sections were used rather than permanent sections. In order to test this and to work out optimal conditions, in the experiment shown, with his help, A549 lung cancer cells were embedded in Matrigel and implanted in athymic nude mice. Two weeks later, before tumors could form, the mice were euthanized, the plugs harvested, and frozen sections made. The sections were then subjected to

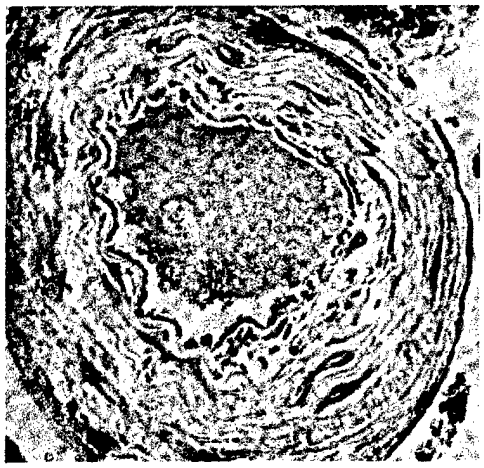

Figure 5. Gax is expressed in both the vascular smooth muscle cells and the endothelial cells of human arteries. A section from human kidney was stained with rabbit polyclonal anti-Gax antibody and counterstained with an antibody linked to horseradish peroxidase.

immunohistochemistry with anti-CD31 antibodies and the vessels growing into the plug examined (Figure 4). Excellent ingrowth of blood vessels was observed.

Based on the results shown in Figure 4, we are now in the process of repeating this experiment in C57BL/6 mice. We are mixing different doses of VEGF, bFGF, and fetal bovine serum in the plugs and implanting them in female C57BL/6 mice. After two weeks, we will harvest the plugs and subject them to frozen section and immunohistochemistry.

Plan for Year Two: We will complete the in vitro experiments examining the effect of various pro- and antiangiogenic agents on *Gax* expression in ECs, as originally proposed in Specific Aim #1. It is fortunate that we began the *in vivo* experiments ahead of schedule. Obtaining adequate neovascularization in the Matrigel plugs (66) has proven more difficult than originally anticipated. At present, we plan on finishing pilot experiments to determine the optimal concentration of VEGF, bFGF, and fetal bovine serum that produces maximal vessel density in the plugs. We will then proceed according to the experimental plan described in Specific Aim #1, implanting Matrigel plugs containing this optimal concentration, harvesting them two weeks later, then measuring *Gax* expression in the Matrigel plug vasculature by immunohistochemistry and real time quantitative PCR.

b. Compare immunohistochemical staining for *Gax* expression in breast tumor blood vessels with that of blood vessels found in normal breast for 50 invasive human breast cancer specimens. (Months 13-36.)

Status: In progress.

Results and Discussion: We have been able to stain the ECs and vascular smooth muscle cells in blood vessels using the polyclonal rabbit anti-Gax antibody (Figure 5). This particular example (normal human kidney from a nephrectomy specimen done for renal cell carcinoma) demonstrates strong staining of the nuclei of the endothelial cells lining the artery, as well as the expected staining of the nuclei of the vascular smooth muscle layer. However, despite this example of good immunohistochemistry

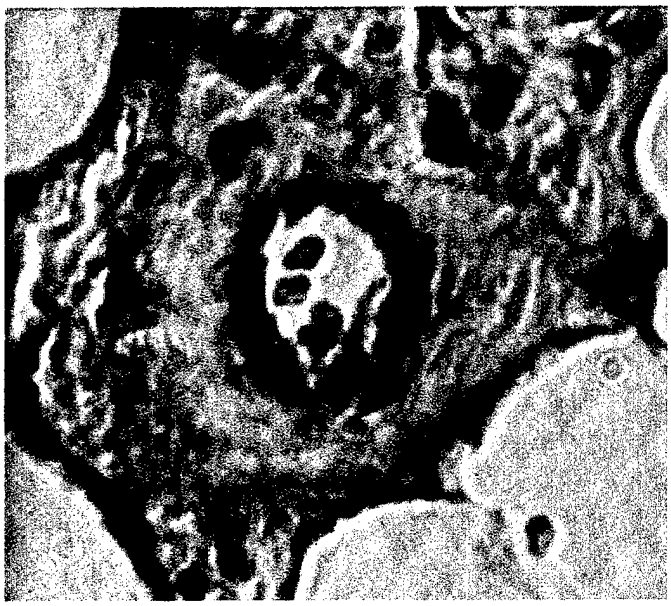

Figure 6. *Gax* expression in tumor blood vessels. PCR was used to generate a 329 bp fragment of the *Gax* cDNA (nucleotides 549 to 877), which was then used to generate a riboprobe for *in situ* hybridization. This probe was used to label sections of normal human colon. This tumor blood vessel stained positive for *Gax* expression. Sense probe did not demonstrate any staining (not shown)

for the *Gax* protein, we have had difficulty obtaining reproducibly good immunohistochemical staining of paraffin-embedded specimens, mainly because of high background staining that has been difficult to eliminate. We are working in collaboration with our Tissue Retrieval Service to optimize conditions further, before using the antibody on clinical breast cancer specimens.

In addition, we have begun pilot experiments to optimize *in situ* hybridization for *Gax* expression. PCR was used to generate a 329 bp fragment of the *Gax* cDNA (nucleotides 549 to 877) (58), which was then used to generate a riboprobe for *in situ* hybridization. In collaboration with Dr. Nancy Boudreau, this probe was used to label sections of a human colon specimen (Figure 6). This normal blood vessel stained positive for *Gax* expression, and the control sense probe did not demonstrate any staining (not shown). We are presently optimizing hybridization conditions for paraffin-embedded tissue, so that we can begin performing *in situ* hybridizations on clinical breast cancer specimens as proposed in Specific Aim #1, in order to study differences in *Gax* expression between normal and breast cancer vasculature.

Plan for Year Two: We plan to continue to optimize our technique for immunohistochemistry and *in situ* hybridization for *Gax*. Once that is achieved, we will begin immunohistochemical staining and *in situ* hybridization of clinical breast cancer tissue specimens for *Gax* expression in tumor vasculature, as described in Specific Aim #1.

Task 3: ***Determine the effects of Gax overexpression in endothelial cells in vitro. (Months 1-24.)***

a. Determine effect of *Gax* overexpression on endothelial cell proliferation and expression of cell cycle regulatory genes. (Months 1-12.)

Status: In progress.

Results and Discussion: The effect of *Gax* expression on EC proliferation and cell cycle progression have been examined. Adenoviral constructs containing the cDNAs for rat and human *Gax* under the control of the CMV early promoter (Ad.*rGax* and Ad.*hGax*, respectively) have been supplied by Dr. Kenneth Walsh. In order to test the hypothesis that *Gax* expression inhibits proliferation of ECs, HUVECs were sparsely plated on plastic in 12 well plates and then transduced with Ad.*hGax* at an increasing multiplicity of infection (MOI). Viable cells were counted from three wells from each experimental group every 24 hours for four days (Figure 7). Control cells were transduced with Ad.*β-gal*. Up to MOI=1000, Ad.*β-gal* did not inhibit HUVEC proliferation (data not shown). Ad.h*Gax*, on the other hand, inhibited HUVEC proliferation in a dose-dependent fashion when compared with Ad.*β-gal* (Figure 7). Quiescent HUVECs were then

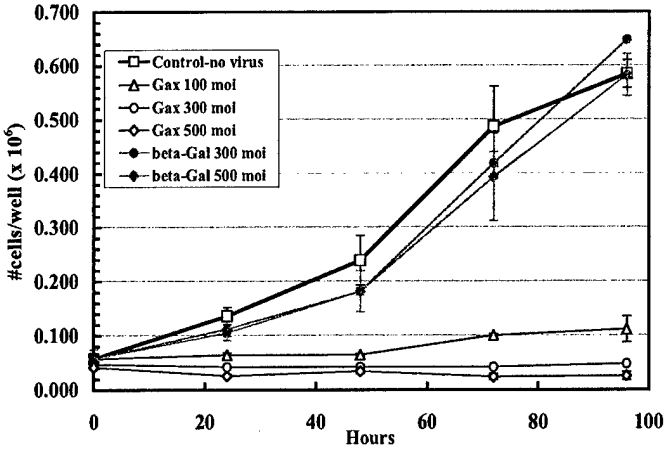

Figure 7. Inhibition of HUVEC proliferation by *Gax*. Randomly cycling HUVECs growing in 6-well plates in EGM-2 medium were infected with varying MOI of either Ad.h*Gax* or Ad.*β-Gal*. After infection, three wells for each experimental group would be trypsinized and counted, with cell viability determined by Trypan blue exclusion, and results counted as mean number of cells ± standard deviation.

transduced with either Ad.*hGax* or Ad.*β-gal*, maintained in low serum medium for 24 hrs, then stimulated with 10% FBS and VEGF=10 ng/ml, and 24 hour ^{3}H-thymidine uptakes measured (Figure 8). Consistent with its effect on randomly cycling cells, *Gax* strongly inhibited mitogen-stimulated ^{3}H-thymidine uptake. Similar results were obtained when HMEC-1 cells were used (data not shown). These results were repeated and confirmed and thus represent a confirmation of preliminary data submitted in the original Career Development Award application. They are included in a manuscript that is presently in press (12).

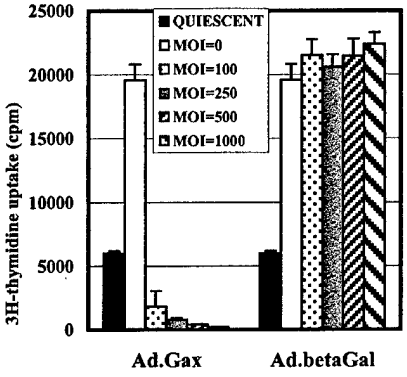

Figure 8. Inhibition of mitogen-induced ^{3}H-thymidine uptake in HUVECs by *Gax*. Quiescent HUVECs were transduced with Ad.*hGax* at various MOI. 24 hrs later, the cells were stimulated with serum and VEGF=10 ng/ml and 24 hr. ^{3}H-thymidine uptakes measured after stimulation.

Based on previous observations that *Gax* inhibits p21 by a p53-independent mechanism in VSMCs and *Gax* expression inhibited HUVEC proliferation as measured both by cell counts and ^{3}H-thymidine uptake, we tested whether *Gax* can induce p21 expression in ECs. HUVECs were transduced with Ad.*hGax* and Ad.*rGax* at varying MOIs. Cells transduced with an adenovirus expressing green fluorescent protein (Ad.GFP) served as controls. By Northern blot, p21 levels were induced in a dose-dependent fashion (Figure 9A) . When cells transduced with Ad.*Gax* in a similar fashion were transfected with a plasmid containing the p21 promoter fused upstream to the firefly Luciferase gene, it was similarly observed that p21 promoter activity was increased by up to 7-fold (Figure 9B). Transduction with Ad.GFP did not alter p21 promoter activity (Figure 9, A and B). These results represent a confirmation of our previously presented preliminary results. Furthermore, the upregulation of p21 by *Gax* expression has been confirmed in our initial cDNA microarray experiments (see Task 5) and by quantitative real time PCR (data not shown). Also, in our preliminary microarray experiments, we have identified other cell cycle genes that appear to be regulated by *Gax* expression (see Task 5). We are currently working to confirm that these genes are indeed regulated by *Gax* and are not artifacts of microarray experiments. Finally, using flow cytometry, we are presently working on confirming that *Gax* induces G_1 cell cycle arrest in ECs, as it does in VSMCs.

Plan for Year Two: Based on our results with p21 and microarray experiments, we plan to conclude this series of experiments, proceeding as described in Specific Aim #2. We will clarify in what phase of the cell cycle arrest occurs in *Gax*-transduced cells. We have also identified p19INK4D as another cyclin kinase inhibitor that, in our initial experiments, appears to be upregulated by *Gax* expression (see Task 5).

a. **Determine effect of *Gax* overexpression on expression of integrins, specifically if the expression of integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ are regulated by *Gax* expression (Months 6-18.)**

Status: In progress.

Results and Discussion: We have recently begun experiments to determine whether *Gax* expression changes the integrin expression profile of vascular ECs. We began by first determining whether *Gax* expression inhibits EC migration towards mitogenic or pro-angiogenic

stimuli. We found that *Gax* strongly inhibited EC migration through polycarbonate membranes towards serum (Figure 10) and also towards VEGF and bFGF (not shown). However, initial experiments using flow cytometry and antibodies directed at specific integrins have not shown a difference in the expression of integrin subunit β_3 or β_5, nor in the overall expression of integrins $\alpha_V\beta_3$ or $\alpha_V\beta_5$ (data not shown), which is different from what is observed in vascular smooth muscle cells when *Gax* expression is driven by these viral constructs(28). One explanation for this difference is that we have not found the optimal conditions to demonstrate this difference. Another possibility is that *Gax* downregulates integrin expression in ECs only in the presence of specific factors that we have not identified yet. Alternatively, it is possible that there is no difference, and that the effects of *Gax* on integrin expression observed in VSMCs are cell type-specific. Given our preliminary observations in Task 5 (Specific Aim #3), it may be that *Gax* inhibits angiogenesis through different cell adhesion molecules, such as E-selectin (see Task 5).

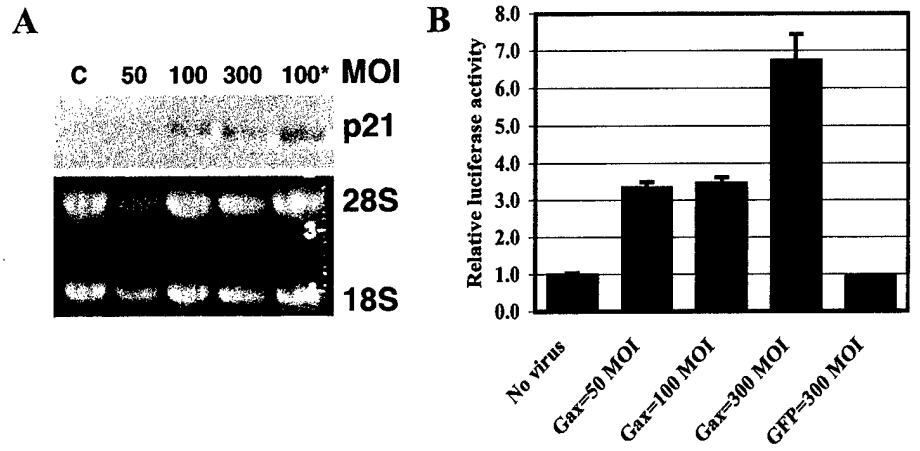

Figure 9. ***Gax overexpression induces p21 expression.*** **A. *Gax* expression induces p21 expression in HUVECs.** Randomly cycling HUVECs were infected with either Ad.*hGax* at varying MOIs , Ad.*rGax* at MOI=100(*), or Ad.*GFP*=300 MOI (**C**) and then were harvested 24 hours later, and Northern blots performed for p21. **B. *Gax* expression induces p21 promoter activity.** HUVECs were infected with Ad.*rGax* and then transfected with a plasmid containing the p21 promoter driving the Firefly Luciferase gene. Luciferase activity was measured 24 hours later and normalized to Renilla Luciferase activity. Error bars represent standard deviation of three wells.

Plan for Year Two: These experiments are ongoing. We plan to survey the effect of *Gax* expression on the expression of integrins known to be involved in regulating angiogenesis by flow cytometry and Western blot. We will do the same for EC adhesion molecules identified in Specific Aim #3 (Task 5). We will then, when possible, determine if inhibition of migration and tube formation by *Gax* can be overcome by overexpressing any of these adhesion molecules.

c. Characterize *Gax*-induced endothelial cell apoptosis and the effect of *Gax* on the expression of genes regulating apoptosis. (Months 13-24.)

Status: Not done.

Plan for Year Two: These experiments, as proposed in Specific Aim #2, are planned for Year Two.

Task 4: ***Determine the effects of Gax overexpression on angiogenesis in vivo. (Months 13-36.)***

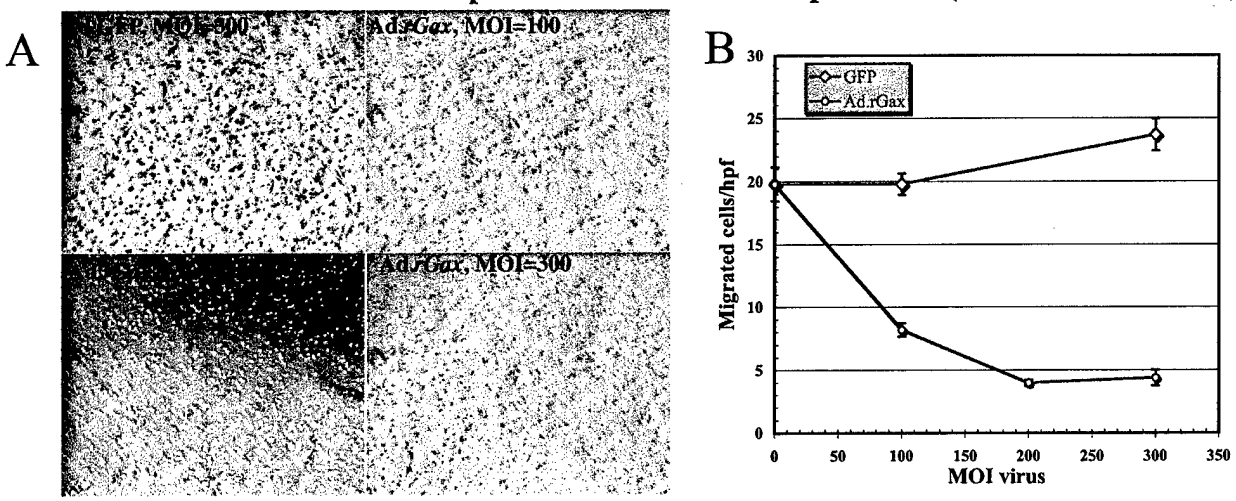

Figure 10. Gax inhibits HUVEC migration towards serum. HUVECs were transduced with varying MOI of either Ad.*GFP* or Ad.*rGax*. After incubation overnight, they were trypsinized, and viable cells counted using Trypan Blue exclusion. 10^5 cells per well were plated in medium containing 0.1% FBS in 6 well plates containing inserts with 8 μm pores and allowed to migrate towards the bottom chamber, which contained 10% FBS. After six hours, the remaining cells were scraped from the 0.1% FBS side with a cotton swab, and the filter was fixed and stained with Dif-Quik. At least 10 high-powered fields were counted per well. Similar results were obtained with Ad.*hGax* (data not shown)

a. Matrigel plug assays in C57BL/6 mice to determine if Ad.*Gax* inhibits *in vivo* angiogenesis and to quantify how strong the effect is. 100 mice will be required. (Months 13-36)

Status: In progress

Results and Discussion: See Task 2a.

Plan for Year Two: As stated in our report on the status of Task 2a (see above), producing adequate neovascularization in the Matrigel plugs (66) has proven more difficult than originally anticipated. At present, we plan on finishing pilot experiments to determine the optimal concentration of VEGF, bFGF, and fetal bovine serum that produces maximal vessel density in the plugs. We will then proceed according to the experimental plan described in Specific Aim #2, implanting Matrigel plugs containing this optimal concentration plus a varying number of pfus of control virus and Ad.*Gax*, harvesting them two weeks later, then subjecting the plugs to immunohistochemistry with anti-CD31 antibody. We will then compare vessel counts in plugs containing Ad.*Gax* with those containing control virus and thereby determine if *Gax* expression inhibits *in vivo* angiogenesis.

Given the difficulties we have had in producing adequate neovascularization in Matrigel plugs without using live tumor cells to maintain a constant production of proangiogenic factors (Figure 4), we are now considering alternative models to answer the question of whether *Gax* inhibits *in vivo* angiogenesis. In our opinion, most promising among these models is an implantable matrix model developed by Nor *et al.* (51). In their model, human dermal microvascular endothelial cells (HDMEC) transplanted into severe combined immunodeficient (SCID) mice on biodegradable polymer matrices differentiated into functional human microvessels that anastomosed with the mouse vasculature. ECs became organized into empty tubular structures by Day 5 and differentiated into functional microvessels within 7 to 10 days.

Moreover, the human microvessels expressed physiological markers of angiogenesis, including CD31, CD34, vascular cellular adhesion molecule-1 (VCAM-1) (67-70), and intercellular adhesion molecule-1 (ICAM-1) (67, 68, 71-74). The human ECs even became invested by perivascular smooth muscle α-actin–expressing mouse cells 21 days after implantation. The advantages of this model include (1) use of human endothelial cells and (2) the ability to genetically manipulate the ECs and observe the effects of this manipulation on *in vivo* angiogenesis. Even better, transient gene expression for less than two weeks would be adequate for this assay. However, once we have succeeded in constructing stable transfectant HMEC-1 cells engineered for tetracycline-inducible *Gax* expression, we could use them in this model. Matrices containing these cells could be implanted in nude mice and the mice fed either a control diet or a diet containing doxycycline, and then differences in vessel counts within the matrices determined. Moreover, we could also use immunohistochemistry to study *in vivo* changes in EC gene expression caused by the induction of *Gax* expression. Thus, we plan on developing this model in our laboratory, in collaboration with Dr. Nancy Boudreau, who is already collaborating with us by providing aid in performing in situ hybridizations and who has already successfully developed the model of *Nor et al.* (51) in her laboratory (9). If we are unable to develop the Matrigel plug model, which still has the advantage of using the less expensive C57BL/6 mouse strain, rather than requiring the use of immunodeficient mice, such as SCID mice or nude mice, we will redirect our effort into this model during Year Two.

b. Chick chorioallantoic membrane assays to quantify *Gax* inhibition of angiogenesis. (Months 13-36.)

Status: Not done.

Plan for Year Two: We will perform these experiments in Year Two, as outlined in Specific Aim #2.

Task 5: ***Identify potential downstream targets of Gax. (Months 1 through 24.)***

a. Construct stably transfected endothelial cells with tetracycline-inducible *Gax* expression and verify inducible *Gax* expression. (Months 1 to 9.)

Status: In progress

Results: We have inserted the cDNA for the human homologue of *Gax* into the pTRE vector (Clontech) in both the forward and reverse orientation and in transient transfection assays verified its doxycycline-inducible *Gax* expression using cell lines provided by the manufacturer. We then measured the sensitivity of HMEC-1 cells to the two selection agents necessary for producing the double stable transfectants, G418 and hygromycin. We found that 400 μg/ml G418 and 200 μg/ml hygromycin were the lowest concentrations required to kill 100% of the HMEC-1 cells tested (data not shown). We therefore started our first stable transfection using the pTet-On plasmid (Clontech) and G418 400 μg/ml. Because previous experiments by the PI, both at The Cancer Institute of New Jersey and the University of Chicago have shown that liposomal-based transfection methods are toxic to ECs (data not shown), we used SuperFect™ reagent (Qiagen), which, in our hands, has been less toxic. In our first two experiments, we have failed to generate a single colony of stably transfected HMEC-1 cells.

Plan for Year Two: Given our inability thus far to generate the needed stable transfectants, we are now reassessing every aspect of our experimental approach to this problem.

First, we are repeating experiments to determine the minimum concentrations of G418 and hygromycin necessary to kill 100% of the HMEC-1 cells. Second, we are examining other stable EC cell lines to use to make stable transfectants, such as HPMEC-ST1.6R, a microvascular endothelial cell line with more of the phenotypic characteristics of primary culture ECs than HMEC-1s (75). Third, we are considering resequencing the plasmids, into which we had previously inserted *Gax*, to verify they are correct. Finally, we are also considering alternate methods of developing stable transfectants with inducible *Gax* expression, including the GeneSwitch™ system (Invitrogen, Carlsbad, CA). This system activates transcription in the presence of subphysiologic concentrations of mefepristone, a progesterone antagonist, and is silent in its absence.

b. Compare global gene expression between *Gax*-expressing endothelial cells and non-*Gax*-expressing endothelial cells using cDNA microarrays. (Months 10 to 18.)

Status: In progress.

Results and Discussion: We are unable to accomplish this task until Task 5a is successfully completed. Because we have not yet been able to construct the stable transfectants with tetracycline-inducible *Gax* expression, in order to make progress in identifying potential changes in global gene expression induced by *Gax* expression, we adopted an alternate technique. We compared global gene expression in control HUVECs infected with an adenoviral construct expressing green fluorescent protein (GFP) with HUVECs infected with Ad.r*Gax*, which expresses the rat homolog of *Gax*. Cells were infected with control virus or Ad.r*Gax* at an MOI=100, incubated 24 hours in normal media, then harvested for RNA isolation. Strong expression of r*Gax* was verified by Western blot using our anti-Gax antibody (13, 30). The quality of the total RNA isolated was checked by running it on a denaturing agarose gel, and then global gene expression compared in two separate experiments using the Affymetrix Human Genome U133A GeneChip® array set and standard protocols supplied by the manufacturer. This gene chip contains probe sets for over 33,000 known genes, along with probes for housekeeping genes for normalization and genomic DNA for evaluation of the quality of hybridization. Although this method has the disadvantage of not allowing study of detailed time courses of global changes in gene expression caused by *Gax* expression, it does allow us to identify promising downstream target genes to evaluate further.

In general, the global changes in gene expression induced by *Gax* in this experiment were consistent with an anti-proliferative, antiangiogenic activity, but some surprising results were also found. We noted that there were probe sets showing an increased signal (0.8% of the total probe sets on the chip) and 229 sets showing a decreased signal (0.7% of the total). Of these, 127 probe sets showing upregulation and 115 showing downregulation corresponded to known genes. Differences in gene expression between controls and *Gax*-transduced cells ranged from upregulation by approximately 30-fold to a downregulation by 238-fold. (However, this downregulation occurred in a gene whose basal expression is at a very low level and may be artifacts.) We began a preliminary survey of genes whose expression was either up- or downregulated in *Gax*-transduced cells, looking for promising downstream targets. As an initial method, using the GO ontogeny tool (76) on the Affymetrix website, we began to categorize these genes into categories by biological function. An initial survey of identifiable genes in which the genes are assigned to functional categories is summarized in Table I.

Table I: Biological functions of genes up- and down-regulated

Functional Category*	No. genes upregulated	No. genes downregulated
Apoptosis (pro- and anti-)	4	7
Cell adhesion/motility	8	19
Cell cycle	5	8
Growth factors	3	6
Hemostasis/coagulation	2	3
Immune function	0	10
Metabolism	27	11
Morphogenesis	13	4
Signal transduction	15	23
Transcription factors	10	15
Miscellaneous	4	2

*Genes may be assigned to more than one functional category.

We then examined the genes in more detail, specifically looking for genes that have either been implicated in EC proliferation or angiogenesis, in an initial attempt to identify such genes that may be regulated by *Gax* expression. Because this represents only two experiments and does not include time courses, this list is not intended to be a definitive list, but rather as a starting point for identifying promising candidate genes to concentrate attention on first, either by real time quantitative PCR or, when antibodies are available commercially, Western blot. Promising potential downstream target genes that were upregulated by *Gax* expression in this experiment are summarized in Table 2; downregulated genes, in Table 3.

Table 2. Genes upregulated 24 hours after *Gax* expression

Gene	Fold increase
MAP4K2	29.9
Bone morphogenetic protein receptor, type IA	27.9
Frizzled	9.8
Proalpha I chain of type I collagen	6.4
ID2	4.6
Thrombomodulin	4.0
p19 (INK4D)	2.5
Angiopoietin-2	2.0

The genes listed in Table 2 were selected based on how strongly they were upregulated after *Gax* expression and for how likely, on initial examination, they were likely to be involved in the mechanism by which *Gax* inhibits in vitro EC proliferation and angiogenesis. For instance, p19 (also called p19INK4D) is a cyclin kinase inhibitor belonging to the INK4 family, and it can be postulated that upregulating both p19INK4D and p21 (member of the *Cip/Kip* family of cyclin kinase inhibitors) provide for redundant mechanisms to induce G_1/S cell cycle arrest (77-80). Next, angiopoietin-2 has been implicated in inhibiting postnatal angiogenesis through its ability to inhibit the activity of angiopoietin-1 on the Tie2 receptor, although under some circumstances and at high concentrations angiopoietin-2 appears to promote angiogenesis, especially in the presence of VEGF (81). It is thus unclear whether angiopoietin-2 is a net inducer or repressor of angiogenesis or whether it depends upon other factors. Similarly, although little is known about the roles of ID proteins in angiogenesis, what is known suggests that they are more likely to be proangiogenic (82, 83). *ID2*, specifically, has been noted to be upregulated in ECs forming tubes (84). However, this increase was noted 18 hours after plating, at which time tubes are mature and ECs have become completely quiescent. Finally, it is interesting to note that our results also hint that TGF-β (bone morphogenetic protein receptor type 1A) and the Wnt signaling pathway (*Frizzled*) may be involved in the downstream effects of *Gax* expression in ECs. We are presently using the GeneMAPP and MAPPFinder software packages (85) to identify patterns of gene expression relative to specific signaling pathways. Further, we are presently in the process of repeating these experiments, in order to obtain large enough data sets with enough repetitions for cluster analysis (86).

Table 3. Genes downregulated 24 hours after *Gax* expression

Gene	Fold decrease
GRO1/α (melanoma growth-stimulated activity alpha)	238.0
E-selectin	60.0
Gax/Mox-2	14.0
VCAM-1	13.0
Flt-4 ligand (VEGF-C)	5.3
Phospholipase-C epsilon	4.3
Ephrin A1	3.0
Fibroblast growth factor-2 (basic)	2.8
Intracellular adhesion molecule-1 (ICAM-1)	2.5
Endothelin-1	2.5
Endothelial-specific molecule-1 (ESM-1)	2.1

Next, we examined genes that were downregulated 24 hours after transduction of HUVECs with Ad.r*Gax*. First, we noted that the human homolog of *Gax* itself was strongly downregulated 24 hours after expression of the rat homolog of *Gax*. We point out that the primers and probe we use for real time quantitative PCR of human *Gax* do not crossreact with

the rat *Gax* (data not shown), implying that there may be a negative feedback mechanism regulating *Gax* expression. Next, we note a very strong downregulation of GRO-1/α, a growth factor for melanoma that has also been implicated in promoting angiogenesis (87-90). However, the basal level of expression was low, and it remains to be determined whether this downregulation has real physiologic effects. we noted the downregulation of several adhesion molecules that are known to be upregulated in ECs during angiogenesis and whose soluble forms have been implicated in inducing angiogenesis (71, 91), including VCAM-1 (67-70), intercellular adhesion molecule-1 (ICAM-1) (67, 68, 71-74), and E-selectin (71, 74, 91-93). Similarly, pro-angiogenic peptides such as bFGF (42, 94, 95), endothelin-1 (96-100), ephrin-A1 (101-105), and VEGF-C (106-108) are also downregulated in *Gax*-transduced ECs, implying a role for *Gax* in decreasing autocrine stimulation of angiogenesis. Finally, endothelial-specific molecule-1 (ESM-1), an EC-specific protein of unknown function that is upregulated in ECs made to form tubes on reconstituted basement membrane (84), is also downregulated. All of these molecules have been implicated in EC activation and angiogenesis (42, 67-74, 84, 91-108), whose downregulation may thus represent potential parts of the mechanism by which *Gax* may inhibit angiogenesis.

Plan for Year Two: We are presently using the GenMAPP and MAPPFinder software packages (85) to identify patterns of gene expression relative to specific signaling pathways. Further, we are presently in the process of repeating these experiments, so that we can develop large enough data sets with enough repeat experiments for cluster analysis. As soon as Task 5a is accomplished, we will begin to analyze the effects on global gene expression of inducing *Gax* expression in ECs in a more rigorous fashion. By treating our control and tetracycline-inducible *Gax*-expressing constructs with doxycycline, we will analyze time courses of induction of various downstream targets of *Gax*, something we cannot do effectively using our adenoviral constructs. Also, using cells with inducible *Gax* expression will greatly reduce the possibility of artifacts caused by genes that may be induced by adenoviral infection. In the meantime, we will verify the regulation promising downstream target genes identified in our initial cDNA microarray experiment by quantitative real time PCR and, for genes for which good quality antibodies are available, Western blot. During the second and third years, we will focus on these genes found on our initial survey to be most likely to be involved in regulating angiogenesis, determining whether they are regulated at the level of transcription or mRNA stability.

c. Suppression subtractive hybridization of RNA from *Gax*-expressing and non-*Gax*-expressing stable transfectants to generate clones of putative *Gax*-regulated genes. (Months 10 to 18.)

Status: Not done

Plan for Year Two: We are two months behind schedule to start these experiments and therefore plan to start these experiments as soon as possible. Once again, this Task depends upon the accomplishment of Task 5a and cannot begin until Task 5a is successfully accomplished. It may not be possible to reach the appropriate milestone at 18 months. If this happens, we will negotiate a change in our Statement of Work to accommodate changes

d. Data analysis of cDNA microarray data to identify putative downstream targets of *Gax*. (Months 19-24.)

Status: In progress.

Results and Discussion: See discussion of Task 5b.

Plan for Year Two: As discussed in Task 5b, we are presently using the GenMAPP and MAPPFinder software packages (85) to identify patterns of gene expression relative to specific signaling pathways and plan to use this data and analysis to identify putative downstream targets and signaling pathways through which *Gax* exerts its effect. See Task 5b for more details.

Task 6: ***Verification that putative downstream targets of Gax identified by cDNA microarray and subtractive hybridization are regulated by Gax. Months 25 through 36.***

a. Sequencing of clones derived from suppression subtractive hybridization. (Months 19-36.)

Status: Not done.

Plan for Year Two: We plan to begin these experiments in the next six months.

b. Northern and Western blots of genes identified in Task 6 in order to verify regulation by *Gax*. (Months 19-36.)

Status: Not done

Plan for Year Two: We will perform these experiments beginning in Year Two, as described in Specific Aim #3.

c. Promoter mapping experiments to identify *Gax*-responsive elements in selected downstream targets of *Gax*. (Months 19-36.)

Status: Not done.

Plan for Year Two: We will perform these experiments beginning in Year Two, as discussed in Specific Aim #3.

KEY RESEARCH ACCOMPLISHMENTS

Our key research accomplishments during the past year include:

1. Developing a reliable and reproducible quantitative real time PCR assay for *Gax*, β-actin, GAPDH, and a number of other genes. These methods allow us to rapidly design new primer/probe sets for genes identified in our cDNA microarray experiments in order to verify that their expression is induced or repressed by *Gax* expression.
2. Demonstrating that mitogens and proangiogenic factors regulate *Gax* expression in ECs in a manner similar to that observed in vascular smooth muscle cells, with its expression maximal in quiescent cells and rapidly downregulated after ECs are treated with mitogens, VEGF, or bFGF.
3. Performing two cDNA microarray experiments and beginning analysis of the data, which presently suggests that *Gax* downregulates the expression of EC adhesion molecules, such as VCAM-1, E-selectin, and ICAM-1, known to be expressed by angiogenic ECs.
4. Confirming that *Gax* expression in ECs inhibits proliferation, cell cycle progression, and induces p21, trans-activating the p21 promoter.
5. Confirming that *Gax* expression inhibits EC tube formation on reconstituted basement membrane.

6. Showing that *Gax* expression inhibits EC migration towards serum and proangiogenic stimuli.

REPORTABLE OUTCOMES

Abstracts

1. **Gorski, D. H.** (2002) The homeobox gene *Gax* induces p21 expression and inhibits vascular endothelial cell activation. *Ann. Surg. Oncol.* 9:S42. (Presented at the Society of Surgical Oncology Meeting, 2002).

Journal articles accepted for publication:

1. Gorski, D. H. and Leal, A. J. Inhibition of endothelial cell activation by the homeobox gene *Gax. J. Surg. Res.* (in press), 2003.
2. Gorski, D. H. and Walsh, K. Control of vascular cell differentiation by homeobox transcription factors. *Trends Cardiovasc. Med.* (in press), 2003.

Funding applied for based on work done on this project:

DAMD17-03-1-0292 **U.S. Department of Defense Breast Cancer Research Program Idea Award** **Role: Principal investigator** *Inhibition of breast cancer-induced angiogenesis by a diverged homeobox gene* **Status: Funded**	4/14/2003 - 4/13/2006 $455,000 (total direct + indirect costs)	40% (No salary support)
DAMD17-03-1-0292 **U.S. Department of Defense Breast Cancer Research Program Idea Award** **Role: Co-Investigator (PI: James Goydos)** *Expression of vascular endothelial growth factors-C and -D (VEGF-C and VEGF-D) and stage in patients with breast cancer* **Status: Submitted.** **Note:** The techniques developed in this project to use quantitative real time PCR allowed us to propose to use QRT-PCR to propose other breast cancer-related projects using QRT-PCR.	4/1/2004 - 3/31/2007 $455,000 (total direct + indirect costs)	10%
New Jersey Commission on Cancer Research **Role: Principal investigator** *Regulation of breast cancer-induced angiogenesis by a homeobox gene* **Status: Recommended for funding. Funding declined, because of excessive overlap with DAMD17-03-1-0292.**	6/1/2003 – 5/31/2005 $99,000	30% (No salary support)

Kimmel Foundation for Cancer Research	7/1/2003 –	40% (No
Program: Kimmel Scholar Award	6/30/2005	salary
Role: Principal investigator	$200,000	support

Regulation of tumor-induced angiogenesis by a homeobox gene

Status: On alternate list for funding.

CONCLUSIONS

The primary target of proangiogenic factors secreted by breast cancer cells, and many antiangiogenic factors, is the vascular endothelial cell. During angiogenesis, whether physiologic or breast cancer-induced, endothelial cells undergo distinct changes in phenotype and gene expression, including activation of proteolytic enzymes to degrade basement membrane, sprouting, proliferation, tube formation, and production of extracellular matrix . Endothelial proliferation accompanies cell invasion and migration, and lumens of new capillaries are formed when endothelial cells adhere to one another and form tubes (39-41). Homeobox genes are master regulatory genes with diverse functions in many cell types, both during embryogenesis and in the adult (1, 3, 4, 6, 109). It is therefore not surprising that recently they have been implicated as important transcriptional regulators controlling endothelial cell phenotype during tumor-induced angiogenesis (7-9, 11, 49, 53).

Until recently, little was known about how homeobox genes might influence endothelial cell phenotype and behavior during breast cancer-induced angiogenesis. However, evidence for their involvement in the phenotypic changes endothelial cells undergo during angiogenesis is now accumulating. For instance, Patel *et al* reported an endothelial cell-specific variant of *HOXA9* whose expression is regulated by tumor necrosis factor-α, which is proangiogenic (110). More direct evidence for the importance of homeobox genes in angiogenesis exists for *HOXD3* (7). *In vivo*, sustained expression of *HOXD3* on the chick chorioallantoic membrane (CAM) retains endothelial cells in an invasive state and prevents vessel maturation, leading to vascular malformations and endotheliomas. In diabetic mice, *HOXD3* expression is impaired in endothelial cells, as is its upregulation after wounding (49). More recently, overexpression of another homeobox gene, *HOXB3* has been shown to result in an increase in capillary vascular density and angiogenesis, and its blockade by antisense results in impaired capillary morphogenesis (8). In contrast, *HOXD10* inhibits EC conversion to the angiogenic phenotype, and sustained expression of *HOXD10* inhibits EC migration and blocks bFGF- and VEGF-induced angiogenesis *in vivo* (51). Another homeobox gene, *Hex,* has a more complex role, being upregulated during angiogenesis but inhibiting EC tube formation on basement membranes (11). When combined with previous data showing high levels of *Hex* expression in proliferating vasculature had suggested that *Hex* would be more likely to induce EC proliferation and angiogenesis (52, 53), the observation that *Hex* inhibits *in vitro* angiogenesis suggests a more complex role for this gene than previously understood. Taken together, these data suggest significant roles for specific homeobox genes in responding to extracellular signals and activating batteries of downstream genes to induce or inhibit the phenotypic changes in endothelial cells associated with angiogenesis. These observations are what initially led us to look for additional homeobox genes likely to be involved in the final transcriptional control of genes determining angiogenic phenotype in breast cancer.

In our work on this project during Year One, we have produced data strongly suggesting a role for another homeobox gene, the growth arrest homeobox gene *Gax*, in regulating the phenotypic changes that occur in vascular endothelial cells during breast cancer-induced angiogenesis. Moreover, unlike cell cycle regulators such as p21 or p53, the expression of this gene is relatively restricted to the cardiovascular system (13, 14). We suspected such a role for *Gax* in endothelial cells during breast cancer-induced angiogenesis because of its activities in vascular smooth muscle cells, which include G_1 cell cycle arrest; p21 activation (30); and inhibition of migration towards cytokines and mitogens. We therefore looked for its expression in vascular endothelial cells using RT-PCR, Northern blot, and immunohistochemistry and found that *Gax* is indeed expressed in endothelial cells, both *in vitro* and *in vivo* in normal human blood vessels. Moreover, its expression blocks endothelial cell proliferation, with this inhibition being associated with an upregulation of p21 (Figure 9). This upregulation is proportional to the level of expression of *Gax*, and appears to be due to activation of the p21 promoter.

We then studied how *Gax* expression is regulated in ECs by mitogens and pro-angiogenic factors and are in the process of studying how *Gax* expression is regulated by breast cancer-secreted factors. The results of these experiments demonstrate that *Gax* expression is rapidly downregulated in ECs after they are exposed to serum, bFGF, or VEGF in a time course and magnitude consistent with its regulation in VSMCs (13, 27). Moreover, *Gax* expression inhibits VSMC migration towards serum and VEGF (Figure 10), consistent with our observation that it inhibits in vitro angiogenesis. In order to look for potential downstream targets, we began cDNA microarray experiments designed to look for differences in gene expression when *Gax* expression is driven in HUVECs. We found several potential downstream targets whose expression level was changed 24 hours after *Gax* expression and that could therefore be molecular mediators that allow *Gax* to inhibit the angiogenic phenotype in response to proangiogenic factors. Genes downregulated included EC-specific adhesion molecules known to be upregulated during angiogenesis and pro-angiogenic growth factors, such as GRO-1 (87-90), VEGF-C (107, 111-113), and bFGF (42, 94, 114, 115), whereas genes upregulated include genes such as the cyclin kinase inhibitor p19INK4D (78-80, 116), the *Tie2* antagonist angiopoietin-2 (81, 117-120), and the bone morphogenetic receptor, type 1A (121, 122). The regulation of most of these genes are consistent with the observed physiologic effects of exogenous *Gax* expression. It remains for Years Two and Three to determine which genes of these are truly up- or downregulated by *Gax* and which of these genes mediates the activity of *Gax*.

Unlike other homeobox genes implicated in regulating the angiogenic, to the best of our knowledge *Gax* is the only gene whose expression is relatively restricted to the cardiovascular system in the adult. This is in marked contrast to *HOXD3*, *HOXB3*, or *HOXA10*, whose expression is widespread (7-9, 49), and also in contrast to *Hex*, whose expression is more restricted, but not confined to the cardiovascular system (53, 123-125). Our data demonstrate that *Gax* is expressed in vascular endothelium, that its expression is regulated by mitogens, and that in this cell type it has activities similar to those in reported VSMCs (13, 27, 29). Based on this rationale and the data from our studies thus far, we propose a model (Figure 11). In this model, proangiogenic factors secreted by breast cancer cells and endogenous antiangiogenic factors bind to various receptors and activate multiple cell signaling pathways. These pathways intersect in the nucleus, where, depending upon the balance of the signals from pro- and antiangiogenic factors (angiogenic balance) (126), *Gax* expression is either activated or inhibited, and it is the activation or downregulation of *Gax* expression that is an important determinant of the angiogenic phenotype. We will now investigate whether expression of *Gax* in ECs can block

angiogenesis. Results of our studies will determine the role of *Gax* in regulating EC phenotype during breast cancer-induced angiogenesis, identify upstream regulators and downstream targets of *Gax* expression and activity. These studies are likely to uncover previously unknown molecular mechanisms of breast cancer-induced angiogenesis controlled at the level of transcription.

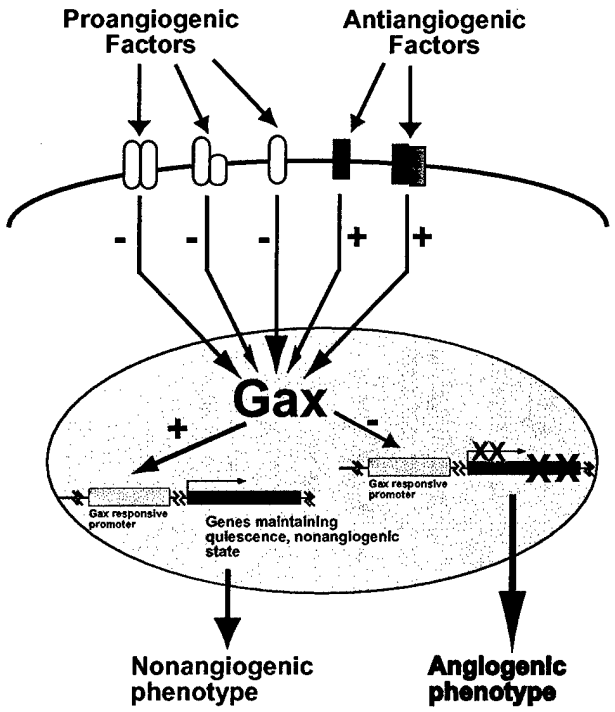

Figure 11. Model for the role of *Gax* in regulating EC phenotype during angiogenesis. We propose that *Gax* acts as a negative regulator of the angiogenic phenotype whose activity and expression depend upon the balance of pro- and anti-angiogenic factors and the signaling pathways they act through. See text for further description.

Breast cancer angiogenesis represents a promising new target for therapy. Given that the most important cell in this process is the vascular endothelial cell, targeting breast cancer-induced angiogenesis implies targeting vascular endothelial cell processes important to angiogenesis. Specific transcription factors such as *Ets-1* are known to integrate the signals coming from the pathways activated by pro- and anti-angiogenic factors and translate these signals to changes in endothelial cell gene expression and phenotype (64, 65). As such, endothelial cell transcription factors represent both a tool for understanding the phenotypic changes endothelial cells undergo in response to proangiogenic factors secreted by breast cancer cells that result in angiogenesis and potential targets for the anti-angiogenic therapy of cancer. We conclude from our studies thus far that *Gax* may represent an important negative regulator of angiogenesis in vascular endothelial cells, and as such may represent a new molecular tool to understand the transcriptional control of changes in gene expression that occur in endothelial cells during breast cancer-induced angiogenesis and, more importantly, a potential target for the antiangiogenic therapy of breast cancer. Future studies funded by this Career Development Award (DAMD17-02-1-0511) in Years Two and Three will determine whether this initial conclusion is correct and begin to show how *Gax* and its downstream targets may serve as starting points for developing angiangiogenic therapies of breast cancer.

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APPENDICES

Publications during period of report:

1. Gorski, D. H. and Leal, A. J. Inhibition of endothelial cell activation by the homeobox gene *Gax. J. Surg. Res.* (in press), 2003.
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Inhibition of endothelial cell activation by the homeobox gene *Gax*

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ABSTRACT

Background: Angiogenesis is critical to tumor growth. *Gax*, a homeobox transcription factor whose expression in the adult is restricted mainly to the cardiovascular system, strongly inhibits growth factor-stimulated phenotypic modulation of vascular smooth muscle cells *in vitro* and *in vivo*. The function of *Gax* in vascular endothelium is unknown, but we hypothesized that it may play a similar role there. We therefore studied *Gax* expression in vascular endothelial cells and its effects on proliferation and tube formation.

Materials and Methods: *Gax* expression in normal endothelial cells was examined *in vitro* by Northern blot and reverse transcriptase polymerase chain reaction, and *in vivo* by immunohistochemistry. A replication-deficient adenovirus was then used to express *Gax* in human umbilical vein endothelial cells (HUVECs). HUVEC proliferation, ^{3}H-thymidine uptake, p21 expression, and tube formation on reconstituted basement membrane were measured at different viral multiplicities of infection.

Results: *Gax* mRNA was detected in HUVECs by RT-PCR and Northern blot analysis and in normal vascular endothelium by immunohistochemistry. Compared to controls transduced with a virus expressing β-galactosidase, *Gax* strongly inhibited HUVEC proliferation and mitogen-stimulated ^{3}H-thymidine uptake. p21 expression in HUVECs transduced with *Gax* was increased up to five-fold as measured by Northern blot, and p21 promoter activity was activated by four- to five-fold. Tube formation on Matrigel was strongly inhibited by *Gax* expression.

Conclusions: *Gax* is expressed in vascular endothelium and strongly inhibits endothelial cell activation in response to growth factors and tube formation *in vitro*. These observations suggest that *Gax* inhibits endothelial cell transition to the angiogenic phenotype in response to

proangiogenic growth factors and, as a negative regulator of angiogenesis, may represent a target for the antiangiogenic therapy of cancer.

KEY WORDS: ANGIOGENESIS, HOMEOBOX GENES, TRANSCRIPTION FACTORS, VASCULAR ENDOTHELIUM

INTRODUCTION

Vascular remodeling plays a critical role in the biology of tumors, whose growth without a blood supply is limited to less than 1 mm in diameter by diffusion of oxygen and nutrients through the interstitial fluids (1). To overcome this limitation, tumors secrete pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) (2) and basic fibroblast growth factor (bFGF) (3), to stimulate the ingrowth of new blood vessels (1, 4). In order to form new tumor vasculature, endothelial cells undergo profound phenotypic changes, many of which are similar to the phenotypic changes tumor cells undergo when invading the surrounding stroma (1, 5, 6). They degrade their basement membrane and invade the surrounding tissue, migrate towards the proangiogenic stimulus secreted by the tumor, and then form tubular structures and finally neovasculature (1, 7). Although the receptors and signaling pathways activated by proangiogenic factors and cytokines have been extensively studied in endothelial cells (8, 9), much less is known about the molecular biology of the downstream transcription factors that regulate the tissue-specific gene expression controlling endothelial cell growth and differentiation and are activated by these signaling pathways. These transcription factors represent a common mechanism that can be influenced by the interaction of multiple signaling pathways and therefore might represent targets for the antiangiogenic therapy of cancer.

To understand the transcriptional control of tumor-induced angiogenesis and thereby potentially identify new ways to target it therapeutically, we decided to study the role of homeobox transcription factors in regulating the phenotypic changes that occur in endothelial cells when stimulated with proangiogenic factors. Because of their ubiquitous role as regulators of cell proliferation, migration, and differentiation, as well as body plan formation and organogenesis during embryogenesis in vertebrates and invertebrates (10, 11) and as oncogenes

and tumor suppressors in various human cancers (12, 13), of all the various classes of transcription factors, we considered homeobox genes as especially likely to be important in regulating endothelial cell phenotype during angiogenesis.

Among homeobox genes, *Gax* (**G**rowth **A**rrest-specific homeobo**X**) has several characteristics that suggest it as a candidate for a role as an inhibitor of the endothelial cell phenotypic changes that occur as a result of stimulation by proangiogenic factors. Originally isolated from vascular smooth muscle (14), in the adult *Gax* expression is largely restricted to the cardiovascular system (14, 15). In vascular smooth muscle cells, *Gax* expression is downregulated by mitogens (14, 16) and upregulated by growth arrest signals (14, 17). Consistent with this observation, *Gax* expression induces G_1 cell cycle arrest (18) and inhibits vascular smooth muscle cell migration, downregulating the expression of integrins, $\alpha_V\beta_3$ and $\alpha_V\beta_5$ (19), both of which are associated with the synthetic state in vascular smooth muscle cells and the angiogenic phenotype in endothelial cells (19, 20). *In vivo*, *Gax* expression in arteries inhibits proliferative restenosis of the arterial lumen after injury (21). Because *Gax* expression is largely confined to the cardiovascular system and mesoderm-derived structures (15, 22), we considered it likely that *Gax* is also expressed in endothelial cells, because endothelial cells are also derived from mesoderm. Because of its activities in vascular smooth muscle cells, we further hypothesized that *Gax* may be involved in inhibiting the phenotypic changes that occur in endothelial cells in response to stimulation with proangiogenic factors. In this report, we show that *Gax* is also expressed in vascular endothelial cells and inhibits endothelial cell cycle activation and tube formation in response to proangiogenic factors, suggesting that it has a role as a negative regulator of angiogenesis.

MATERIALS AND METHODS

Cells and cell culture

Human umbilical vein endothelial cells were obtained from BioWhittaker (Walkersville, MD) and cultured as previously described (23) according to manufacturer's instructions in EGM-2 medium (BioWhittaker, Walkersville, MD). For experiments, recombinant $VEGF_{165}$ (R & D Systems, Minneapolis, MN) was substituted in the media at the concentrations indicated for the proprietary VEGF solution.

Plasmid and adenoviral constructs

The *Gax* cDNA was maintained in pBluescript SK+ vectors and excised as needed for use as probes for Northern blots. Adenoviral constructs expressing the rat and human homologs of *Gax* (Ad.*hGax* and Ad.*rGax*, respectively) conjugated to the α-hemagluttinin (HA) epitope were a kind gift of Dr. Kenneth Walsh (Boston University, Boston, MA) (18), as was the control adenoviral vector expressing β-galactosidase (Ad.*β-Gal*). Both human and rat isoforms of Gax were used, in order to verify that both isoforms have similar activity. The control adenoviral vector expressing green fluorescent protein (Ad.*GFP*) was a kind gift of Dr. Daniel Medina (The Cancer Institute of New Jersey, New Brunswick, NJ). Viral titers were determined by plaque assay. Prior to the use of Ad.*hGax* or Ad.*rGax* in HUVECs, expression of *Gax* mRNA and protein in cells transduced with these adenoviral constructs were verified by Northern and Western blot (not shown). The p21 cDNA and p21 promoter constructs were also obtained from Dr. Kenneth Walsh, and are the same constructs used in (18). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA used as a probe for Northern blots was the same construct used in (14).

Immunohistochemistry

Tissue sections were obtained from human surgical specimens and fixed and imbedded in paraffin according to standard procedures, with sections dehydrated through xylenes and then rehydrated through graded ethanols (15). Staining with a polyclonal rabbit anti-Gax antibody, which labels rat, human, and mouse Gax protein, was performed according to previously described methods, except that the dilution used was 1:1000 (15). A biotin-labeled goat anti-rabbit IgG (Sigma Corporation, St. Louis, MO) was used as a secondary antibody, and Gax staining was visualized using Vectastain ABC (Vector Laboratories, Burlingame, CA). Background staining was assessed by staining sections without primary antibody. All tissue specimens were obtained from a protocol approved by the Institutional Review Board of the University that protects the privacy of the patients from which the samples were obtained.

Northern blots

Northern blots measuring *Gax* expression were performed as previously described (14). Briefly, total RNA (30 μg) was isolated from cultured cells using the guanidinium thiocyanate method (24), subjected to electrophoresis through formaldehyde-containing agarose gels, capillary blotted to nylon membranes using 10X SSC as the transfer buffer, fixed to the membrane using ultraviolet crosslinking, and then hybridized to the *Gax* cDNA labeled with ^{32}P by random priming in Church buffer (25). Blots were exposed to Kodak XAR-5 X-ray film with an intensifying screen at –80° C. Blots were then stripped with 0.1X SSC plus 0.1% SDS at 95° C and reprobed with the GAPDH cDNA to verify equal RNA loading. Hybridization temperatures were 55° C for *Gax*, p21, and GAPDH probes, and all blots were washed to a stringency of 0.2X SSC at 65° C. For p21 Northern blots, autoradiographs were scanned and

band intensities determined with NIH Image v.1.6. p21 message levels were then normalized to GAPDH levels, and the fold-induction of p21 determined.

Reverse transcriptase polymerase chain reaction

RNA was isolated as described above from HUVECs and used in reverse transcriptase polymerase chain reactions (RT-PCR) to detect *Gax* transcripts. Total RNA (5 μg) was subjected to reverse transcriptase reaction with MMLV-reverse transcriptase (Invitrogen, Carlsbad, CA) using random hexamers (Invitrogen, Carlsbad, CA). Because *Gax* has a single exon (26), all samples were treated with RNAse-free DNAse I (Ambion, Austin, TX) prior to being subjected to reverse transcription. As a further means of verifying that there was no genomic DNA contamination, control reactions with no reverse transcriptase were also subjected to PCR. To check the integrity of the RNA, the same reverse transcriptase reactions used to detect *Gax* were subjected to PCR using β-actin-specific primers. Human *Gax* primer sequences were: 5'-GTCAGAAGTCAACAGCAAACCCAG-3', sense; 5'-CACATTCACCAGTTCCTTTTCCCGAGCC-3', antisense; product size 247 bp, from nucleotides 566 to 812 (26). Human β-actin primer sequences were: 5'-ATCCGCAAAGACCTGT-3', β-actin sense; and 5'-GTCCGCCTAGAAGCAT-3' β-actin antisense; product size 270 bp, from nucleotides 906 to 1175 (27). Before *Gax* primers were synthesized, their sequences were subjected to a BLAST (28) search against the Genbank database, in order to detect any possibility that they might bind to or amplify genes other than *Gax*. Before running assays on experimental samples, each primer set, annealing conditions, Mg^{2+} concentration, and primer and probe concentration were optimized using plasmids containing the cDNA of interest. 25 μl reaction mixtures were used containing 0.75 U *Taq* polymerase (Gibco BRL), reaction buffer, 0.2 mM dNTPs, plus the optimized concentrations of

$MgCl_2$, probe, and primers for each primer set. The PCR cycle consisted of an initial 5 minute denaturation step at 95° C, followed by 35 cycles of denaturation at 95° C for 30 seconds, annealing at 56° C (*Gax*) or 54° C (β-actin) for each primer for 60 seconds, and extension at 72° C for 60 seconds.

Cell proliferation and ^{3}H-thymidine incorporation

The effect of *Gax* overexpression on mitogen-stimulated ^{3}H-thymidine incorporation was examined in HUVECs. For cell proliferation, randomly cycling HUVECs in six-well plates (20,000 cells/plate) were transduced for 12 hours with Ad.*Gax* or Ad.*β-gal* at varying MOIs, after which they were washed three times with PBS and then placed in fresh medium EGM-2 supplemented with 10 ng/ml $VEGF_{165}$). After infection, every day three wells for each experimental group were trypsinized and viable cells counted, with cell viability determined by Trypan blue exclusion. For ^{3}H-thymidine uptake studies, HUVECs were made quiescent by serum starvation for 16 hours in medium containing 0.1% FBS, at which point the cells were transduced with Ad.*Gax* or Ad.*β-Gal* and incubated in 0.1% FBS for an additional 24 hours. The cells were then stimulated with medium containing 10% FBS and 10 ng/ml $VEGF_{165}$ for 24 hours in the presence of 0.2 μCi/ml ^{3}H-thymidine (Amersham, Piscataway, NJ), after which trichloroacetic acid precipitable counts were measured.

Transactivation of the p21 promoter

Subconfluent HUVECs were plated in six-well plates and allowed to attach for 4 hours. They were then infected with different MOIs of Ad.*hGax* or Ad.*GFP* overnight, then transfected with p21 promoter Luciferase reporter construct. Transfection was carried out using 2 μg p21-Luciferase plasmid per well, plus 0.2 μg pRL-SV (Promega, Madison, WI), which contains the cDNA for *Renilla reniformis* Luciferase downstream from the SV40 promoter as its reporter

instead of the cDNA for firefly Luciferase as a control for transfection efficiency. Firefly and *Renilla* Luciferase activities were measured using the Dual Luciferase Assay Kit (Promega, Madison, WI), and the firefly Luciferase activity from the p21-Luciferase promoter construct normalized to the constitutive *Renilla* Luciferase activity from the pRL-SV plasmid.

Tube formation assay

Tube formation assays were carried out essentially as described (29). Briefly, HUVECs were infected with adenoviruses expressing either human *Gax* (Ad.*hGax*), rat *Gax* (Ad.*rGax*), or *GFP* (Ad.*GFP*) at various MOI. 18 hours later 5 x 10^5 cells were plated on six-well plates whose surfaces had been coated with reconstituted basement membrane, Low Growth Factor Matrigel, (BD Biosciences, San Jose, CA) and incubated overnight in the presence of serum and 10 ng/ml $VEGF_{165}$. After this, the number of tubes per high-powered field were counted for ten high-powered fields, with tubes being defined as a completed connection between cells. Ad.GFP-transduced cells were also examined using a fluorescence microscope to demonstrate that GFP was being expressed in the HUVECs forming tubes.

Data analysis and statistics

Experiments were repeated three or more times. For cell culture experiments, at least three wells per experimental group were measured and the mean ± standard deviation determined. Statistical significance between the various groups was determined by two-way ANOVA and the appropriate post-test, with the results being considered statistically significant when $p<0.05$.

RESULTS

Gax *is expressed in human vascular endothelium*

Because we hypothesized that Gax is expressed in endothelial cells as well as vascular smooth muscle cells, we first examined *Gax* expression in cultured human vascular endothelial cells and detected *Gax* expression in HUVECs by Northern blot (Figure 1A) and by RT-PCR using human *Gax*-specific primers (Figure 1B). Next, to verify that Gax protein is expressed in the endothelium of normal human blood vessels, we subjected a section of human kidney from a nephrectomy specimen to immunohistochemistry with a polyclonal rabbit anti-Gax antibody (15) (Figure 2). As expected, *Gax* was strongly expressed in vascular smooth muscle cells. In addition, it was also expressed in the endothelial cells lining the lumen of arteries, as evidenced by nuclear staining of the cells of the intima. From these observations, we conclude that *Gax* is expressed in normal endothelial cells, both *in vitro* and *in vivo*.

Gax *inhibits HUVEC proliferation* in vitro

To test the hypothesis that *Gax* expression inhibits proliferation of endothelial cells, we transduced HUVECs that had been sparsely plated on plastic in six well plates with Ad.*hGax* at increasing MOI. Viable cells were counted from each experimental group every 24 hours for four days. Control cells were transduced with Ad.*β-gal*. Up to MOI=1000, Ad.*β-gal* did not inhibit HUVEC proliferation (data not shown). Both Ad.*hGax* and Ad.*rGax*, on the other hand, inhibited HUVEC and proliferation in a dose-dependent fashion compared to Ad.*β-gal* (Figures 3, A and B; $p<0.05$ for all MOI of virus). Quiescent HUVECs were then transduced with either Ad.*hGax* or Ad.*β-gal*, maintained in low serum medium for 24 hrs, then stimulated with 10% FBS and $VEGF_{165}$=10 ng/ml, and 24 hour ^{3}H-thymidine uptakes measured (Figure 4). For comparison, one experimental group was left in low serum medium and is labeled "Quiescent."

Consistent with its effect on randomly cycling HUVECs. *Gax* strongly inhibited mitogen-stimulated ^{3}H-thymidine uptake ($p<0.05$ for all MOI of virus). From these results, we conclude that *Gax* expression results in inhibition of HUVEC proliferation, as well as cell cycle arrest.

Gax *activates p21 promoter activity in endothelial cells*

Because *Gax* induces p21 in vascular smooth muscle cells and *Gax* expression inhibited HUVEC proliferation as measured both by cell counts and ^{3}H-thymidine uptake, we tested whether *Gax* can induce p21 expression in endothelial cells. HUVECs were transduced with Ad.*hGax* and Ad.*rGax* at varying MOIs. Cells transduced with an adenovirus expressing green fluorescent protein (Ad.*GFP*) served as controls. By Northern blot, p21 levels were strongly induced in a viral MOI-dependent fashion (Figure 5A) . When cells transduced with Ad.*hGax* in a similar fashion were transfected with a plasmid containing the p21 promoter fused upstream to the firefly Luciferase gene, it was similarly observed that p21 promoter activity was increased by up to 7-fold (Figure 5B; $p<0.05$ for all MOI). Transduction with Ad.*GFP* did not affect p21 promoter activity (Figure 5, A and B), nor did transduction with Ad.*β-Gal* (data not shown).

Gax inhibits endothelial cell tube formation on reconstituted basement membranes

We next studied the effect of *Gax* expression on angiogenesis *in vitro*. HUVECs were transduced with Ad.*hGax* and Ad.*rGax* at varying MOIs and plated on reconstituted basement membrane (Matrigel) in the presence of serum and 10 ng/ml $VEGF_{165}$, conditions that result in robust tube formation. Ad.*GFP* had no effect on tube formation up to MOI=250, and expression of GFP was verified by fluorescence microscopy (Figure 6). However, there was a dose-dependent decrease in tube formation beginning at relatively small doses of virus (MOI=25) and becoming maximal at MOI=100 (Figures 6). Maximal inhibition occurred at a much lower MOI than is necessary to maximally inhibit endothelial cell proliferation and activate p21 expression

and became maximal at MOI=50 to 100. We note that is the dose range of virus that we have determined to be necessary to transduce 100% of HUVECs (not shown), implying that few viral particles per cell are necessary to produce sufficient Gax protein to inhibit the cellular machinery that causes tube formation. This is in contrast to the much higher viral MOI necessary to produce maximal inhibition of cell cycle progression and induction of p21 expression, implying that more viral particles per cell and therefore a higher level of Gax protein are required to mediate these effects.

DISCUSSION

The primary target of proangiogenic factors secreted by tumor cells, and many antiangiogenic factors, is the vascular endothelial cell (1, 30). During angiogenesis, whether physiologic or tumor-induced, endothelial cells undergo distinct changes in phenotype and gene expression, including activation of proteolytic enzymes to degrade basement membrane, sprouting, proliferation, tube formation, and production of extracellular matrix (1, 4, 31). Endothelial proliferation accompanies cell invasion and migration, and lumens of new capillaries are formed when endothelial cells adhere to one another and form tubes. Homeobox genes are master regulatory genes with diverse functions in many cell types, both during embryogenesis and in the adult (10-13). It is therefore not surprising that recently they have been implicated as important transcriptional regulators controlling endothelial cell phenotype during angiogenesis.

Until recently, little was known about how homeobox genes might influence endothelial cell phenotype and behavior during angiogenesis. However, evidence for their involvement in the phenotypic changes endothelial cells undergo during angiogenesis is now accumulating. For instance, Patel *et al* reported an endothelial cell-specific variant of *HOXA9* whose expression is regulated by tumor necrosis factor-α, which is proangiogenic (32). More direct evidence for the

importance of homeobox genes in angiogenesis exists for *HOXD3*. Stimulation of endothelial cells with bFGF induces *HOXD3* expression, as well as integrin $\alpha_V\beta_3$ and the urokinase plasminogen activator, effects that are blocked by *HOXD3* antisense. *In vivo*, sustained expression of *HOXD3* on the chick chorioallantoic membrane (CAM) retains endothelial cells in an invasive state and prevents vessel maturation, leading to vascular malformations and endotheliomas (33). In diabetic mice, *HOXD3* expression is impaired in endothelial cells, as is its upregulation after wounding (34). More recently, overexpression of another homeobox gene, *HOXB3*, in the chick CAM has been shown to result in an increase in capillary vascular density and angiogenesis, and its blockade by antisense results in impaired capillary morphogenesis (35). Taken together, these data suggest significant roles for specific homeobox genes in responding to extracellular signals and activating batteries of downstream genes to induce the phenotypic changes in endothelial cells associated with angiogenesis. These observations are what initially led us to look for additional homeobox genes likely to be involved in the final transcriptional control of genes determining angiogenic phenotype.

In this study, we have reported data strongly suggesting a role for another homeobox gene, the growth arrest homeobox gene *Gax*, in regulating the phenotypic changes that occur in vascular endothelial cells during angiogenesis. Moreover, unlike cell cycle regulators such as p21 or p53, the expression of this gene is relatively restricted to the cardiovascular system (14, 15). We suspected such a role for *Gax* in endothelial cells during angiogenesis because of its activities in vascular smooth muscle cells, which include G_1 cell cycle arrest (18); p21 activation (18); and inhibition of migration towards cytokines and mitogens (19). We therefore looked for its expression in vascular endothelial cells using RT-PCR, Northern blot, and immunohistochemistry and found that *Gax* is indeed expressed in endothelial cells, both *in vitro*

(Figure 1) and *in vivo* in normal human blood vessels (Figure 2). Moreover, its expression blocks endothelial cell proliferation, with this inhibition being associated with an upregulation of p21. This upregulation is proportional to the level of expression of *Gax*, and appears to be due to activation of the p21 promoter.

Tumor angiogenesis represents a promising new target for anticancer therapy. Given that the most important cell in this process is the vascular endothelial cell, targeting angiogenesis implies targeting vascular endothelial cell processes important to angiogenesis. Specific transcription factors such as *Ets-1* (36) are known to integrate the signals coming from the pathways activated by pro- and anti-angiogenic factors and translate these signals to changes in endothelial cell gene expression and phenotype. As such, endothelial cell transcription factors represent both a tool for understanding the phenotypic changes endothelial cells undergo in response to proangiogenic factors secreted by tumor cells that result in angiogenesis and potential targets for the anti-angiogenic therapy of cancer. *Gax* is a homeobox transcription factor originally isolated in vascular smooth muscle cells that has previously been shown to be involved in cardiovascular remodeling (19, 21, 37), inhibiting vascular smooth muscle cell proliferation (18) and migration (19). We have now shown that *Gax* is also expressed in vascular endothelial cells (Figures 1 and 2). Moreover, *Gax* inhibits endothelial cell proliferation (Figures 3 and 4) as well, activating p21 expression (Figure 5). Of most interest, *Gax* also strongly inhibits tube formation on reconstituted basement membranes (Figure 6), suggesting that, in addition to its role in inhibiting vascular smooth muscle cell-dependent vascular remodeling processes such as intimal hyperplasia (18, 19), it may also have a role inhibiting vascular remodeling processes that depend mainly on endothelial cells, such as angiogenesis. We therefore conclude that *Gax* may represent an important negative regulator of angiogenesis in

vascular endothelial cells, and as such may represent a new molecular tool to understand the transcriptional control of changes in gene expression that occur in endothelial cells during angiogenesis and, more importantly, a potential target for the antiangiogenic therapy of cancer.

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FIGURE LEGENDS

Figure 1. ***Gax expression in vascular endothelial cells.*** Total RNA from HUVECs was subjected to Northern blot with the *Gax* cDNA labeled with ^{32}P by random priming. **A. Northern blots.** Two different HUVEC preparations were studied and compared to mouse heart (**MH**), which is known to express *Gax*. **B. RT-PCR.** Total RNA from HUVECs was subjected to RT-PCR using primers that amplify a 247 bp fragment (base 566 to 812) of the human *Gax* cDNA. The same RT reactions were also subjected to PCR using β-actin primers. See **Materials and Methods** for details. (**G=*Gax*; A=β-actin**).

Figure 2. ***Gax is expressed in both the vascular smooth muscle cells and the endothelial cells of normal human arteries.*** A section from human kidney obtained from a nephrectomy specimen for renal cell carcinoma was stained with rabbit polyclonal anti-Gax antibody. In the section containing normal kidney, Gax expression was noted in both the media, containing vascular smooth muscle cells (VSMCs), as expected from previous studies, but there was also strong staining in the endothelial cells (ECs) in the intima lining the lumen.

Figure 3. ***Inhibition of HUVEC proliferation by Gax.*** Randomly cycling HUVECs growing in 6-well plates in EGM-2 medium were infected with varying MOI of either Ad.*hGax*, Ad.*rGax*, or Ad.*β-Gal*. After infection, three wells for each experimental group were trypsinized and counted, with cell viability determined by Trypan blue exclusion, and results counted as mean number of cells ± standard deviation. Inhibition of proliferation was statistically significant for all experimental groups at all time points from 48 hours on ($p<0.05$). **A. Effect of Ad.*hGax* on HUVEC proliferation. B. Effect of Ad.*rGax* on HUVEC proliferation.**

Figure 4. ***Inhibition of mitogen-induced ^{3}H-thymidine uptake in HUVECs by Gax.***

Quiescent HUVECs were transduced with Ad.*hGax* at various MOI. 24 hrs later, the cells were stimulated with serum and $VEGF_{165}$ (10 ng/ml) and 24 hr. ^{3}H-thymidine uptakes measured after stimulation. *Gax* strongly inhibited ^{3}H-thymidine uptake in response to mitogen stimulation.

Figure 5. ***Gax overexpression induces p21 expression.*** **A. *Gax* expression induces p21 expression in HUVECs.** Randomly cycling HUVECs were infected with either Ad.*hGax* at varying MOIs , Ad.*rGax* at MOI=100(*), or Ad.*GFP*=300 MOI (**C**) and then were harvested 24 hours later, and Northern blots performed using a p21 probe. **B. *Gax* expression induces p21 promoter activity.** HUVECs were infected with Ad.*rGax* and then transfected with a plasmid containing the p21 promoter driving the firefly Luciferase gene. Luciferase activity was measured 24 hours later and normalized to *Renilla* Luciferase activity. Error bars represent standard deviation of three wells.

Figure 6. ***Gax inhibits VEGF-induced endothelial cell tube formation on Matrigel.***

HUVECs were infected with adenoviruses expressing either human *Gax* (Ad.*hGax*), rat *Gax* (Ad.*rGax*), or *GFP* (Ad.*GFP*) at the MOI indicated. 18 hours later 5 x 10^{5} cells were plated on Matrigel in six-well plates and incubated overnight in the presence of serum and 10 ng/ml VEGF. Tube formation was strongly inhibited by both Ad.*hGax* and Ad.*rGax* ($p<0.05$ at MOI=25). **A. HUVECs in culture demonstrating the inhibition of tube formation by increasing MOI of Ad.*hGax* and Ad.*rGax*. B. Tube counts for an experiment in which Ad.*hGax* was used to inhibit endothelial cell tube formation.**

Figure 1

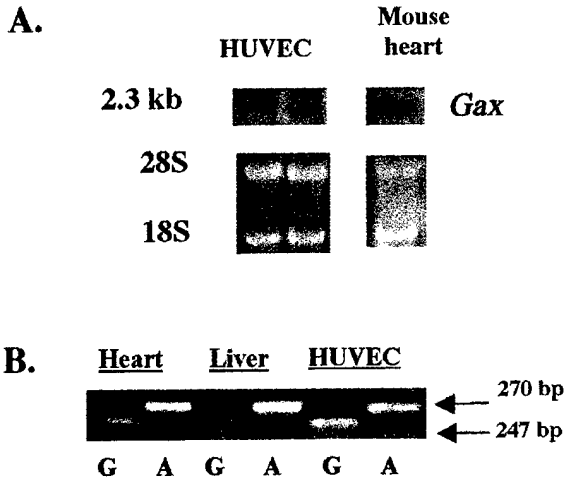

A.
HUVEC
Mouse heart
2.3 kb
Gax
28S
18S
B.
Heart
Liver
HUVEC
270 bp
247 bp
G A G A G A

Figure 2

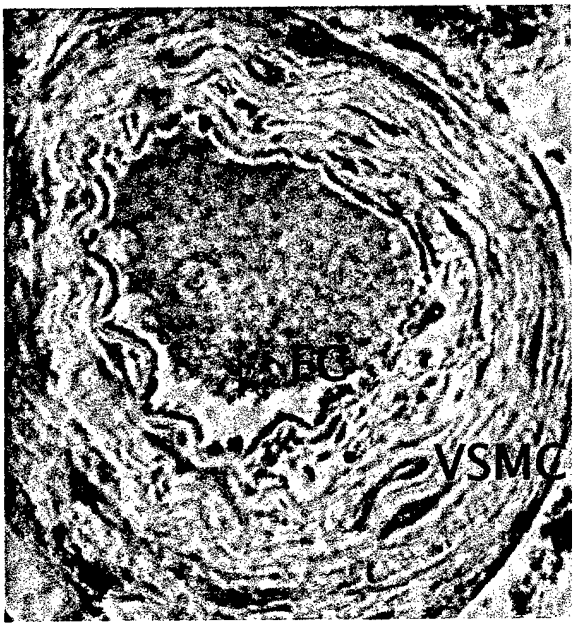


Figure 3A

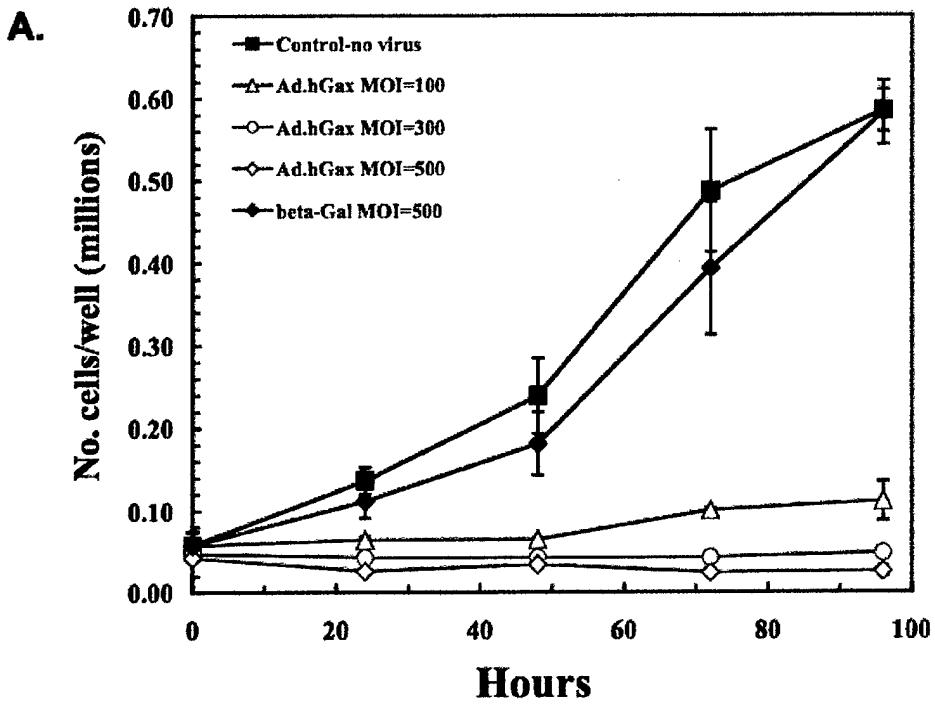

A.
Control-no virus
Ad.hGax MOI=100
Ad.hGax MOI=300
Ad.hGax MOI=500
beta-Gal MOI=500
No. cells/well (millions)
0.00
0.10
0.20
0.30
0.40
0.50
0.60
0.70
0
20
40
60
80
100
Hours

Figure 3B

Figure 3B

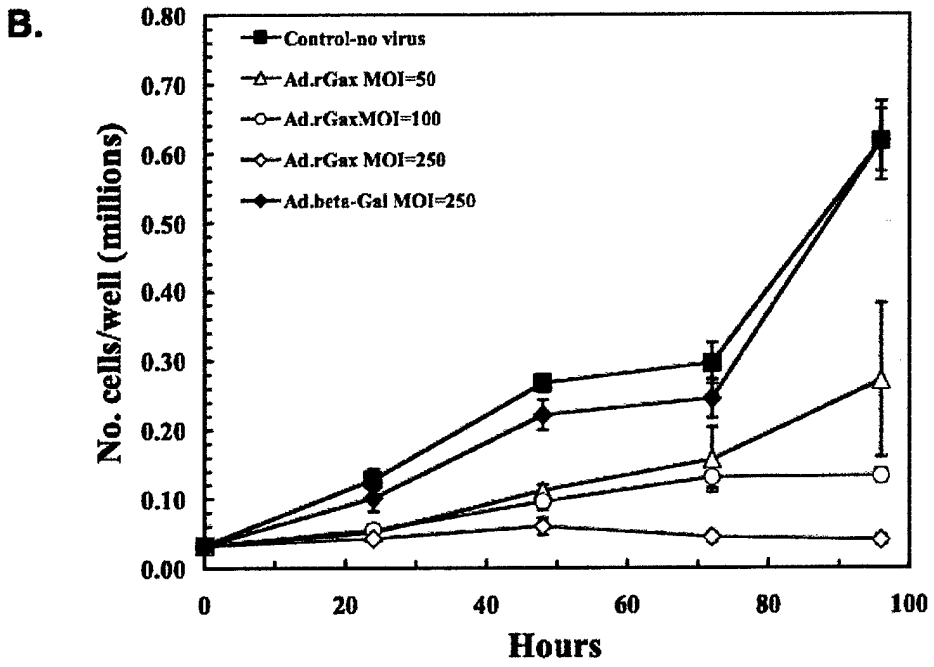


Figure 4

Figure 4

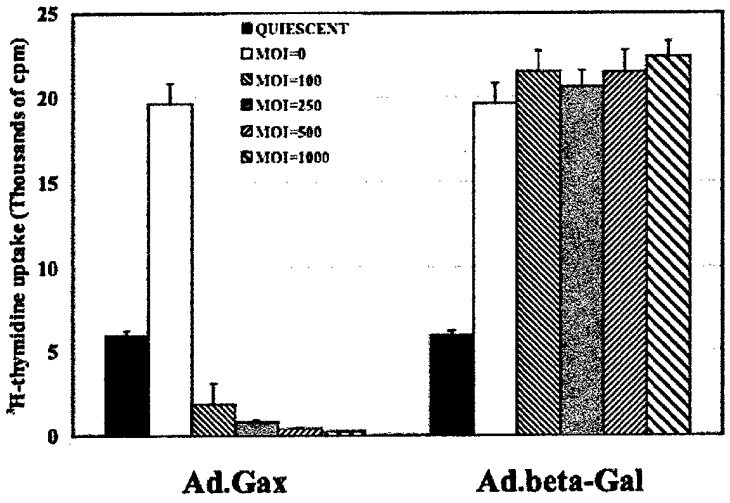


Figure 5A

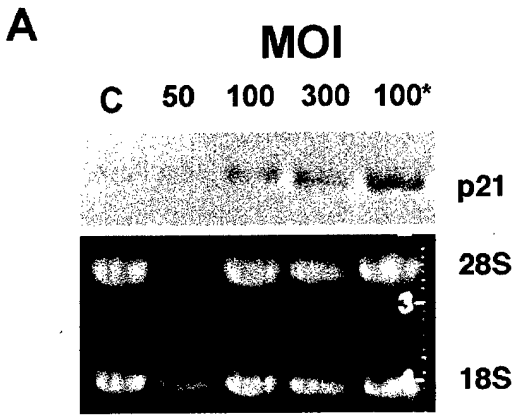


Figure 5B

Figure 5B

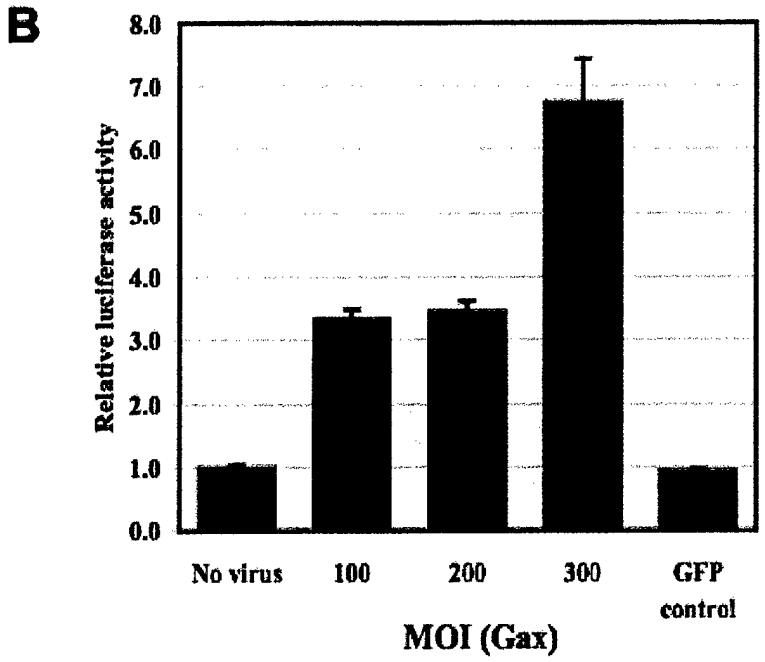


Figure 6A

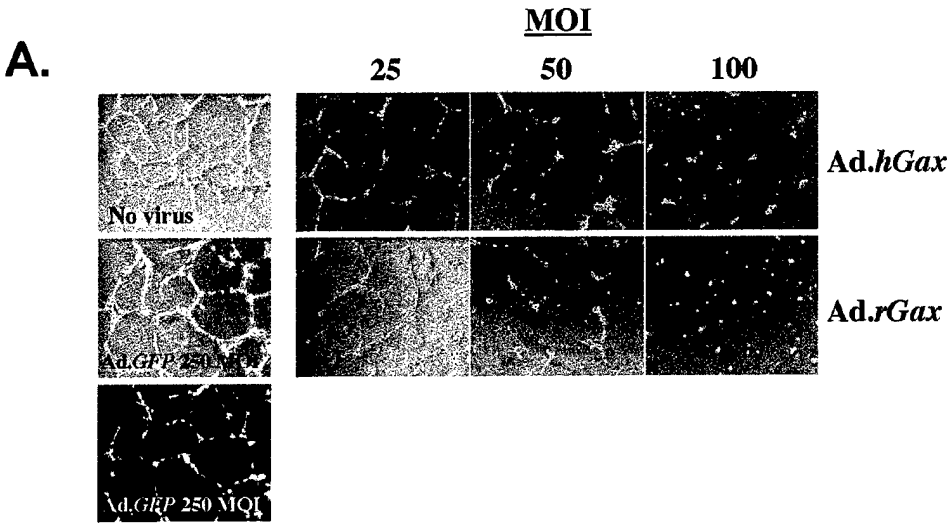
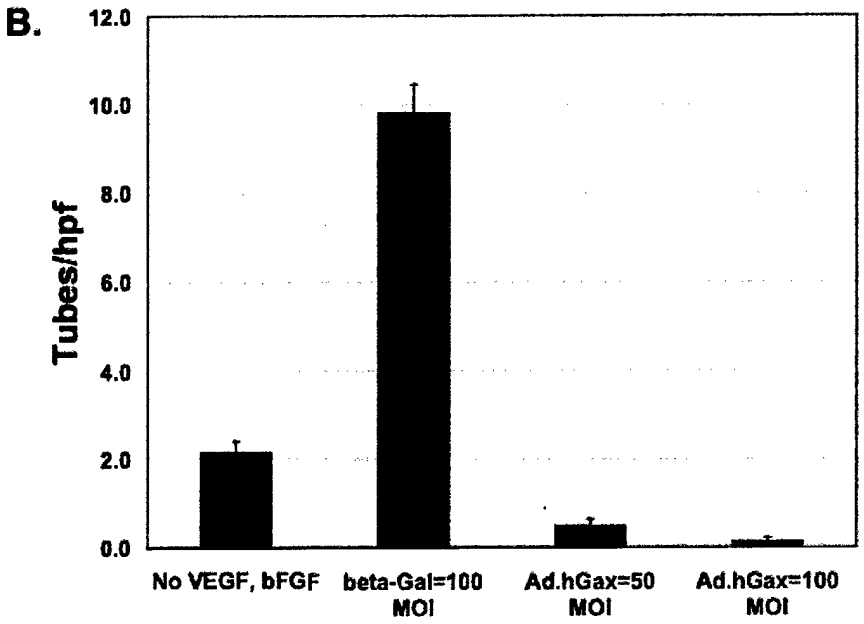


Figure 6B


B.
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Tubes/hpf
No VEGF, bFGF
beta-Gal=100 MOI
Ad.hGax=50 MOI
Ad.hGax=100 MOI

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Control of vascular cell differentiation by homeobox transcription factors

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Running title: *Homeobox genes and vascular differentiation*

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Abstract

Homeobox genes are a family of transcription factors with a highly conserved DNA-binding domain that regulate cell proliferation, differentiation, and migration in many cell types in diverse organisms. These properties are responsible for their critical roles in regulating pattern formation and organogenesis during embryogenesis. The cardiovascular system undergoes extensive remodeling during embryogenesis, and cardiovascular remodeling in the adult is associated with normal physiological processes, such as wound healing and the menstrual cycle, and diseases states, such as atherosclerosis, tumor-induced angiogenesis, and lymphedema. Aside from their roles in the formation of the embryonic vascular system, homeobox genes have recently been implicated in both physiologic and pathologic processes involving vascular remodeling in the adult, such as arterial restenosis after balloon angioplasty, physiologic and tumor-induced angiogenesis, and lymphangiogenesis. Understanding how homeobox genes regulate the phenotype of smooth muscle and endothelium in the vasculature will improve our insight into the molecular mechanisms behind vascular cell differentiation and may suggest therapeutic interventions in human disease.

Introduction

Changes in cellular phenotype leading to remodeling in the vascular system occur during normal development and in pathological states. During embryogenesis, vascular endothelial cell (EC) precursors converge into blood islands, which ultimately develop into the aortic arches and capillary networks that provide oxygen and nutrients to the developing organs and limbs. From this, lymphatic EC precursors bud from embryonic veins to form the lymphatic vascular system. In the adult, examples of changes in vascular cell phenotype leading to vascular remodeling include wound healing and the menstrual cycle, during which both angiogenesis and regression of blood vessels are tightly regulated. Examples of pathological remodeling include atherosclerosis and arterial restenosis after balloon angioplasty. In both processes, vascular smooth muscle cells (VSMCs) migrate from the media to the intima and proliferate, leading to the narrowing of the arterial lumen and the subsequent complications, including hypoxia or even anoxia in downstream tissues (Ross 1993), quickly in the case of restenosis and slowly in the case of atherosclerosis. In addition, phenotypic changes in vascular ECs leading to vascular remodeling play a critical role in tumor biology because diffusion of oxygen and nutrients limits tumor growth to within 1 mm of a capillary. To overcome this limitation, tumors secrete proangiogenic factors to stimulate the ingrowth of new blood vessels (Folkman 1995), which develop from ECs with an immature phenotype (Eberhard et al. 2000). Similarly, tumors also secrete pro-lymphangiogenic factors, which allow the ingrowth of lymphatics and subsequent metastasis to regional lymph nodes (Skobe et al. 2001). Thus, understanding the mechanisms underlying the phenotypic changes that lead to vascular remodeling could produce insights into diseases as diverse as atherosclerosis or restenosis, lymphedema, and cancer.

Although the receptors and signaling pathways activated by growth factors and cytokines have been extensively studied in the vascular system, much less is known about the molecular biology of the downstream transcription factors activated by these pathways to regulate tissue-specific gene expression controlling the growth and differentiation of these cells. Transcription factors represent a common mechanism that can integrate multiple signaling pathways to produce the necessary changes in gene expression and phenotype for vascular cells to perform their functions. Homeobox genes encode a family of transcription factors containing a common 60 amino acid DNA-binding motif known as the homeodomain, containing a helix-turn-helix motif similar to that found in prokaryotic regulatory proteins

such as Cro, CAP, and the λ-repressor in *Escherichia coli* (Scott et al. 1989). They are regulators of cell differentiation, proliferation, and migration in both vertebrates and invertebrates, controlling pattern formation in the embryo and organogenesis, as well as oncogenesis in the adult (Cillo et al. 1999, Ford 1998, Krumlauf 1994). Given these characteristics, they are excellent candidates for important roles in the final transcriptional regulation of genes responsible for vascular remodeling and angiogenesis in normal physiology and disease. Indeed, recently several homeobox genes have been implicated in the phenotypic changes in vascular cells that lead to intimal hyperplasia, arterial restenosis after angioplasty, angiogenesis, and lymphangiogenesis. It is therefore an opportune time to review briefly what is currently known about homeobox gene expression and activity during vasculogenesis and vascular remodeling in the adult.

HOMEOBOX GENE EXPRESSION AND FUNCTION DURING VASCULAR DEVELOPMENT

HOX cluster genes

In *D. melanogaster* and vertebrates, many, but not all, homeobox genes are arranged in gene clusters. In mice and humans, there are four unlinked complexes, HOX A through HOX D, that arose from gene duplication (Krumlauf 1994). Because of this, each HOX gene may have as many as three paralogues. The location of each HOX gene in the cluster corresponds to its axial pattern of expression in the developing embryo, with 5' genes expressed more caudally and 3' expressed more rostral (Fig. 1), with specific embryonic defects due to knockouts of specific HOX genes occurring in the axial region of their expression. HOX genes have been widely studied with regard to their ability to control pattern formation in the developing embryo. They are powerful regulators of pattern formation, as evidenced by the homeotic mutations (i.e., mutations in which one normal body part is substituted for another normal body part, as in *Antennapedia*).

Several members of the HOX clusters are expressed in the cardiovascular system during embryogenesis, including *HOXA5*, *HOXA11*, *HOXB1*, *HOXB7*, and *HOXC9* (Miano et al. 1996). Moreover, there is functional evidence for involvement of HOX genes in vasculogenesis. For example, transgenic mice with null mutations of the *HOXA3* gene die shortly after birth, suffering from defects in the cardiovascular system that include heart wall malformations, persistent patent ductus arteriosus, and

aortic stenosis (Chisaka and Capecchi 1991). In some of these mice, the right carotid artery fails to form, and in all mice the aorta is thin-walled and poorly developed. The overall constellation of defects in *HOXA3* null mice is similar to that observed in the human congenital disorder DiGeorge's syndrome (Emanuel et al. 1999).

Because paralogous HOX genes have similar DNA-binding domains and axial expression patterns during embryogenesis, it has been hypothesized that they may have overlapping or complementary functions. Thus, targeting one paralogue may not produce an observable phenotype. This has been demonstrated by antisense targeting of the messages for the paralogous HOX 3 group (*HOXA3* and *HOXB3*), which results in the regression of aortic arch 3 in a manner similar to that of arch 2 (Kirby et al. 1997). Similarly, targeting paralogous group 5 genes (*HOXA5*, *HOXB5*, and *HOXC5*) causes the appearance of an additional pharyngeal arch containing a novel and aortic arch artery (Kirby et al. 1997). These observation suggest that paralogues probably have overlapping functions in vascular development and that in at least some cases they can compensate for each other when the function of one is impaired.

Paired-related genes

The expression of two genes not located in the HOX clusters, *Prx1* (formerly known as *MHox* or *Phox*) (Cserjesi et al. 1992) and *Prx2* (formerly known as *S8*) (Opstelten et al. 1991), during embryogenesis suggest that they have an important role in vasculogenesis. In the vascular system, expression of *Prx1* and *Prx2* is associated with the primary vessel wall and becomes increasingly restricted to the adventitial and outer medial cell layers as development proceeds (Bergwerff et al. 1998). *Prx1* expression colocalizes with procollagen I and fibrillin-2 but not with smooth muscle α-actin, whereas *Prx2* expression is highly associated with the developing ductus arteriosus and is one of the earliest markers of its differentiation. Transgenic mice with null mutations *Prx1* and *Prx2* suggest their relative importance in vascular patterning in the embryo. $Prx2^{-/-}$ mutants do not show cardiovascular malformations. In contrast, $Prx1^{-/-}$ mutants display abnormal positioning and awkward curvature of the aortic arch, in addition to a misdirected and elongated ductus arteriosus (Bergwerff et al. 2000). However, $Prx1^{-/-}/Prx2^{-/-}$ double mutants demonstrate a more severe form of these abnormalities, some of them possessing an anomalous retro-esophageal right subclavian artery, as well as excessive tortuosity of all great vessels as they run through the mesenchyme, although they do not have cardiac anomalies

(Chesterman et al. 2001). Thus, the loss of *Prx2* function exacerbates anomalies due to the loss of *Prx1*, suggesting functional overlap between these two genes in vascular development.

Hex: An early marker of EC precursors and regulator of EC and VSMC differentiation

Hex is a proline-rich divergent homeobox gene originally isolated from hematopoietic tissues (Crompton et al. 1992). Expressed in a range of hematopoietic progenitor cells and cell lines (Crompton et al. 1992), *Hex* is an early marker of EC precursors and is transiently expressed in the nascent blood islands of the visceral yolk sac and later in embryonic angioblasts and endocardium (Thomas et al. 1998). The *Xexnopus laevis* homologue *XHex* is expressed in vascular ECs throughout the developing vascular network, and its overexpression leads to disruption of vascular structures and an overall increase in EC number (Newman et al. 1997). These observations suggest an important role for *Hex* in the vascular patterning due to the migration and proliferation of EC precursors. In addition, it has been recently reported that *Hex* is also expressed in VSMCs (Sekiguchi et al. 2001). Its expression is upregulated in neointimal VSMCs after balloon injury in the rat, and *Hex* activates the promoter of NMHC-B/SMemb, a non-muscle-specific isoform of the smooth muscle myosin heavy chain that is expressed during embryonic development of the aorta, declines in the neonate and adult, and is re-induced in vascular lesions.

Given the above experimental observations, it has been assumed that *Hex* promotes the conversion of ECs to the angiogenic phenotype. However, recent evidence does not support that assumption and suggests that the role of *Hex* in controlling vascular phenotype may be more complex than first thought. First, disruption of the *Hex* gene in mouse embryos does not produce a detectable change in the vascular phenotype (Barbera et al. 2000), suggesting that other factors, perhaps the transcription factor *Scl* (Liao et al. 2000), may compensate for the loss of *Hex* function. Also, it has recently been reported that *Hex* overexpression in human umbilical vein endothelial cells (HUVECs) inhibits *in vitro* surrogates for angiogenesis, including migration towards vascular endothelial growth factor (VEGF), invasion, proliferation, and tube formation on reconstituted basement membrane (Matrigel) (Nakagawa et al. 2003). In addition, *Hex* was shown to inhibit the expression of angiogenesis-related membrane genes, including those encoding VEGFR-1, VEGFR-2, neuropilin-1, integrin subunit α_V, TIE-1, and TIE-2. It remains to be clarified whether *Hex* inhibits angiogenesis *in vivo*, but, taken together with

previous reports, these observations suggest a complex role for *Hex* in regulating the proliferation and development of the vascular tree and the differentiation of ECs and VSMCs.

Prox1 and development of the lymphatic system

The lymphatic system is a vascular network of thin-walled capillaries and larger vessels lined by a layer of endothelial cells that drain lymph from the tissue spaces of most organs and return it to the venous system for recirculation. Early in development, primitive lymph sacs develop from endothelial budding from the veins to form the lymphatic system. The homeobox gene *Prox1* has been implicated in the development of the lymphatic system. Originally isolated by its homology to the *Drosophila* gene *prospero* (Oliver et al. 1993), *Prox1* has an expression pattern that suggests a functional role in a variety of tissues, including eye lens, central nervous system, and liver, with null mutations leading to embryonic lethality (Wigle and Oliver 1999). Supporting a role in lymphatic development is the observation that *Prox1* is the earliest marker of lymphatic endothelial cell precursors, and in *Prox1*$^{-/-}$ knockout mice budding of endothelial cells that give rise to the lymphatic system is arrested at embryonic day 11.5, resulting in mice without lymphatic vasculature (Wigle and Oliver 1999). In contrast, vasculogenesis and angiogenesis are unaffected by the loss of *Prox1* function (Wigle et al. 2002, Wigle and Oliver 1999). In addition, expression of *Prox1* in vascular ECs results in proliferation and a reprogramming of these cells to a lymphatic EC phenotype, inducing expression of lymphatic genes such as *VEGFR-3*, *p57*^{kip2}, and *desmoplakin I/II* and downregulating vascular EC genes such as *STAT6* and *neuropilin-1* (Hong et al. 2002, Petrova et al. 2002). Moreover, this lymphatic reprogramming due to *Prox1* expression occurs only in vascular ECs, although *Prox1* is still able to induce cyclin expression and proliferation in other cell types (Petrova et al. 2002). Together, these data suggest a role for *Prox1* as a general inducer of proliferation and a key regulatory gene in the developing lymphatic system.

HOMEOBOX GENE EXPRESSION AND FUNCTION IN MATURE BLOOD VESSELS

Homeobox gene expression during VSMC phenotypic modulation and vascular disease

VSMCs exist within a spectrum of phenotypes ranging from the "contractile" to the "synthetic" state (Ross 1993). Cells in the contractile state are quiescent; do not migrate; are relatively insensitive to mitogens; express contractile proteins, including smooth muscle-specific isoforms of actin and myosin;

and are associated with normal vessel wall. Synthetic state cells, on the other hand, are able to migrate; express lower levels of contractile proteins, with higher levels of non muscle isoforms of myosin and actin; secrete extracellular matrix components; and generally resemble less differentiated VSMCs found in fetal blood vessels. Over the last decade, evidence has been accumulating that homeobox genes are involved in regulating the transition between these two phenotypes.

In the adult, several members of the HOX clusters are expressed in the cardiovascular system. Homeobox sequences isolated from adult rat aorta include *HOXA2*, *HOXA4*, *HOXA5*, and *HOXB7*, and *HOXA11* (Gorski et al. 1994, Patel et al. 1992). Other groups have reported the expression of *HOXA5*, *HOXA11*, *HOXB1*, *HOXB7*, and *HOXC9* in human adult and fetal aortic smooth muscle (Miano et al. 1996, Patel et al. 1992). Of these, *HOXB7* and *HOXC9* are expressed at markedly higher levels in embryonic VSMCs compared to adult, suggesting a role in the proliferation and remodeling that occur during embryogenesis (Miano et al. 1996). In addition, overexpression of *HOXB7* in C3H10T1/2 cells results in increased proliferation, the induction of a VSMC-like morphology, and the expression of early, but not intermediate, VSMC markers. In addition, *HOXB7* mRNA was detected in human atherosclerotic plaques at a higher level than in normal human arterial media (Bostrom et al. 2000). These observation suggest a role for *HOXB7* and perhaps *HOXC9* in vascular remodeling, either in the expansion of immature VSMCs or the change of vascular myocytes to a more immature phenotype, both of which occur in human vascular diseases, such as atherosclerosis and restenosis after balloon angioplasty.

Gax and control of smooth muscle phenotype

Originally isolated from a rat aorta cDNA library with the use of degenerate oligonuceotide probes directed at the most conserved protein sequence of the *Antennapedia* homeodomain (Gorski et al. 1993a), *Gax* (also known as *Mox-2*) encodes a homeodomain-containing transcription factor whose expression has multiple effects on vascular phenotype. Although its expression is more widespread in the embryo, including all three muscle lineages and brain (Skopicki et al. 1997), *Gax* expression in the adult is more narrowly confined to cardiovascular tissues, including heart, medial smooth muscle cells of arteries, lung, and mesangial cells in the kidney (Gorski et al. 1993a). In VSMCs, *Gax* expression is rapidly downregulated by mitogenic signals such as serum, platelet-derived growth factor (Gorski et al. 1993a), and angiotensin II (Yamashita et al. 1997), and more slowly upregulated by growth arrest signals,

such as serum deprivation (Gorski et al. 1993a) and C-type natriuretic peptide (Yamashita et al. 1997). Moreover, *Gax* expression is also downregulated in the proliferating VSMCs of the rat carotid artery after balloon injury (Weir et al. 1995). *Gax* expression induces G_0/G_1 cell cycle arrest and upregulates p21 expression by a p53-independent mechanism, and it is this upregulation of p21 that accounts for its antiproliferative activity (Smith et al. 1997). *Gax* also controls the migration of VSMCs towards chemotactic growth factors through its ability to alter integrin expression, downregulating integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ through the specific suppression of the β_3 and β_5 subunits, both *in vitro* and *in vivo* (Witzenbichler et al. 1999). Cell cycle arrest, which does not by itself suppress VSMC migration, is essential for the antimigratory activity of *Gax*, as *Gax* overexpression has no effect on $p21^{-/-}$ cells. Collectively, these data suggest that *Gax* may function to coordinate vascular cell growth and motility through its ability to regulate integrin expression in a cell cycle-dependent manner. The ability of *Gax* to induce apoptosis in proliferating VSMCs (Perlman et al. 1998) is consistent with these observations, as integrin signaling is an important regulator of cell viability.

Control of smooth muscle phenotype by Prx

The expression of *Prx1* and *Prx2* cannot be detected in the vasculature of adult rats, but they are upregulated in rat pulmonary arteries in which pulmonary hypertension was induced by the injection of monocrotaline (Jones et al. 2001). Induction of *Prx1* and *Prx2* expression *in vitro* and *in vivo* is coincident with induction of the extracellular matrix protein tenascin-C, which promotes growth and survival of cultured VSMCs. *Prx1* activates the tenascin-C promoter and induces VSMC proliferation *in vitro*. Consistent with these observations, *Prx1* is upregulated by angiostatin II and, along with the serum response factor, mediates angiotensin II-induced smooth muscle α-actin expression in VSMCs (Hautmann et al. 1997). Collectively, it appears that *Prx1* and *Prx2* genes have roles both in regulating the proliferation of embryonic VSMCs during the formation of the vascular system and in controlling the change of mature VSMCs to a more immature phenotype that occurs in some vascular diseases.

Homeobox genes and post-natal angiogenesis

Functional evidence for the involvement of HOX cluster genes in the regulation of the angiogenic phenotype comes from the study of the paralogous HOX genes *HOXD3* and *HOXB3*, each of which appear to have distinct and complementary roles in this process. *HOXD3* is expressed at high levels in

proliferating ECs induced to form tubes on Matrigel but not in quiescent ECs, and its expression is induced by basic fibroblast growth factor (bFGF) (Boudreau et al. 1997). Functionally, blocking *HOXD3* expression with antisense inhibits the bFGF-stimulated upregulation of integrin $\alpha_V\beta_3$ and urokinase plasminogen activator (uPA) without affecting EC proliferation. In contrast, overexpressing *HOXD3* leads to expression of these genes and a morphologic change to the angiogenic phenotype, resulting in the formation of endotheliomas *in vivo*. In diabetic mice, *HOXD3* expression is impaired in endothelial cells, as is its upregulation after wounding, suggesting that impaired *HOXD3* expression might be involved in the impaired wound healing observed in diabetics (Uyeno et al. 2001). In addition, the *HOXD3* paralogue, *HOXB3*, has been reported to influence angiogenic behavior in a manner distinct from *HOXD3*. Antisense against *HOXB3* impairs the capillary morphogenesis of dermal microvascular ECs and decreases the phosphorylation of the Eph A2 receptor (Myers et al. 2000). Consistent with this result, constitutive expression of *HOXB3* results in an increase in capillary vascular density and angiogenesis but does not produce endotheliomas. Taken together, these results suggest overlapping and complementary roles for *HOXB3* and *HOXD3* in angiogenesis with *HOXD3* promoting the invasive or migratory behavior of ECs in response to angiogenic signals and *HOXB3* promoting capillary morphogenesis of these new vascular sprouts.

In contrast to *HOXB3* and *HOXD3*, another HOX cluster gene, *HOXD10*, inhibits EC conversion to the angiogenic phenotype. Expression of *HOXD10* is higher in quiescent endothelium as compared to tumor-associate vascular endothelium. Moreover, sustained expression of *HOXD10* inhibits EC migration and blocks bFGF- and VEGF-induced angiogenesis in the chick chorioallantoic membrane assay *in vivo*. Consistent with these observations, human ECs overexpressing *HOXD10* fail to form new blood vessels (Myers et al. 2002) when embedded in Matrigel-containing sponges (Nor et al. 2001) in nude mice. In addition, human ECs overexpressing *HOXD10* express a gene profile consistent with a quiescent, nonangiogenic state, with decreased expression of genes that influence remodeling of the extracellular matrix and cell migration during angiogenesis, such as the uPA receptor and the α_3 and β_4 integrin subunits (Myers et al. 2002). Based on these observations, coupled with the proangiogenic activity of *HOXB3* and *HOXD3*, it has been proposed that the 5' and 3' HOX genes have distinct influences on EC

behavior, with the more 3' genes tending to promote the angiogenic phenotype and the more 5' HOX genes such as *HOXD10* tending to be inhibitory to the angiogenic phenotype and dominant.

The expression of other members of the HOX clusters have also been detected in vascular ECs. One example is *HOXA9EC*, an alternatively-spliced variant of HOXA9 whose expression is downregulated by tumor necrosis factor-α (TNF-α) which, in addition to its numerous other activities, is proangiogenic (Patel et al. 1999). In addition, the expression of several members of the HOX B cluster in HUVECs is regulated by VEGF and tissue plasminogen activator, but not bFGF (Belotti et al. 1998). Because HOX B cluster gene expression does not correlate with the mitogenic state of the cell, but rather is altered with the state of cellular differentiation, it has been suggested that these genes are involved in the morphogenic changes associated with the angiogenic phenotype.

Recently, it has been reported that *Gax* is also expressed in vascular ECs (Gorski and Leal 2003). As in VSMCs, in ECs *Gax* expression results in cell cycle arrest and induces p21 expression and promoter activity. Of note, it also strongly inhibits EC tube formation in response to VEGF on Matrigel (Gorski and Leal 2003) in a manner similar to that of *Hex* (Nakagawa et al. 2003). These additional observations suggest that, in addition to its likely role in maintaining VSMCs in the contractile phenotype, *Gax* may also have a role in EC differentiation. Taken together, all the above observations suggest that *Gax* may be a global inhibitor of vascular cell activation. However, like *Hex* knockout mice (Barbera et al. 2000), mice transgenic for a null mutation in *Gax* have not been reported to show vascular anomalies (Mankoo et al. 1999). Rather, they show skeletal muscle anomalies in the limbs and die shortly after birth from unknown causes. This would tend to suggest that other homeobox factors, such as *Mox-1* (Candia and Wright 1996) or possibly *Pax3* (Stamataki et al. 2001), might compensate for a lack of *Gax/Mox-2* expression in the developing cardiovascular system. It would be of great interest to determine whether *Gax* knockout mice demonstrate increased angiogenesis in response to proangiogenic stimuli, but such studies would be difficult because of their very brief lifespan. Similar studies would also be of interest in *Hex* knockout mice.

Other homeobox genes are also likely to be involved in regulating angiogenesis, whether physiologic or tumor-induced. For example, St. Croix *et al.* used serial analysis of gene expression (SAGE) to look for expressed sequence tags (ESTs) whose expression is at least 10-fold greater in tumor

endothelium compared to normal endothelium (St. Croix et al. 2000). Not surprisingly, many of the ESTs they reported derive from extracellular matrix proteins. However, one EST was similar to the homeobox gene *Dlx-3*, a member of the *Distal-less* family of homeobox genes. This EST was not detectable in the developing corpus luteum, implying a distinction between tumor angiogenesis and physiologic angiogenesis. Interestingly, *Dlx-3* has been implicated in placental function (Beanan and Sargent 2000). Other placental homeobox genes include *Dlx-4*, *Gax/Mox-2*, *HB24*, and *Msx2* (Quinn et al. 1997). Given the critical importance of angiogenesis and blood vessel regression in placental function, it is reasonable to predict that some of these genes are involved in vascular remodeling in the placenta. It is also reasonable to postulate that homeobox genes previously demonstrated to be important in inducing proliferation and migration of ECs and EC precursors during angiogenesis, such as *Hex*, may also be important in inducing angiogenesis in the adult vasculature.

CONCLUSIONS

Although there is much more known since the last time we reviewed for this journal what is known about the expression and function of homeobox genes in the vasculature (Gorski et al. 1993b), our knowledge of the transcriptional regulation of VSMC and EC phenotype is still not as detailed as our understanding of the cytokines and growth factors that act upon ECs and VSMCs to regulate their phenotype, the receptors these factors activate, and the downstream signaling pathways activated in turn by these receptors. However, a growing number of homeobox genes have been implicated in vascular development in the embryo and vascular remodeling, angiogenesis, and vascular diseases in the adult. Moreover, with the description of *Prox1* (Hong et al. 2002, Petrova et al. 2002), it has become clear that homeobox genes participate in the development of the lymphatic vascular system as well. Given the sheer number of homeobox genes and potential interactions between them and vascular remodeling, it is difficult to generalize too much about the roles of homeobox genes in these processes, some of which are listed in Table 1. It is, however, possible to come to a few general conclusions regarding how homeobox genes regulate vascular remodeling:

1. Pathways controlled by homeobox genes are redundant, especially during embryogenesis. This implies that it is more likely to be the overall pattern of homeobox gene expression, rather than any one individual homeobox gene, that regulates the phenotype of VSMCs and ECs during

angiogenesis and vascular remodeling. The roles of *HOXB3*, *HOXD3*, and *HOXD10* in regulating EC phenotype during angiogenesis represent a good example of this principle. It may be the balance between pro- and anti-angiogenic HOX cluster genes that determine whether an EC becomes angiogenic or not, and different proangiogenic HOX genes may control different stages or aspects of angiogenesis (*HOXB3* and *HOXD3*, for example). It can also be postulated that *Gax* and *Hex* also help to determine this balance. Similarly, in VSMCs, it could be postulated that the balance between *Gax* and *Prx1*/*Prx2* (and possibly *Hex*) plays a major role in determining whether VSMCs become contractile or synthetic.

2. Individual homeobox genes may function as master regulatory genes for parts of the vascular system. For instance, although a master regulatory gene controlling development of angioblasts into vascular ECs or VSMCs remains to be identified, *Prox1* represents a very good candidate for a such a role in lymphatic endothelium. However, it must be remembered that most homeobox genes controlling vascular phenotype are also expressed in other tissues. Even *Prox1* is expressed in liver and eye lens during embryogenesis. Similarly, *Prx1* is clearly important in skeletal development (ten Berge et al. 1998), and *Gax* is important in skeletal muscle development (Mankoo et al. 1999). This implies that cell type-specific factors influence the activities of homeobox genes in both ECs and VSMCs and that homeobox genes may be downstream from other, more global, master regulatory genes. Indeed, *Prox1* can only reprogram a vascular EC to take on the phenotype of lymphatic endothelium (Petrova et al. 2002). It cannot so reprogram other cell types.
3. Little is known about how homeobox genes implicated in angiogenesis and vascular remodeling exert their effects at the molecular level. However, it is clear that at least a subset of them appear to function by controlling the differentiation, proliferation, and/or migration of VSMCs and ECs. The mechanism behind these phenotypic changes must be the activation and repression of specific batteries of downstream genes. Because few downstream genes from homeobox genes are known, one of the most fertile areas of research for homeobox gene research is the identification of their downstream targets and the elucidation of the mechanisms by which homeobox genes regulate the expression of these target genes and these target genes in turn lead to the phenotypic changes observed. In the near future, it is likely that cDNA microarray technology will provide an excellent tool

for identifying the global changes in gene expression occurring in response to homeobox gene expression in vascular cells.

Given their importance in cell cycle control, cell migration, and cell adhesion, it is likely that many more homeobox genes will be implicated in the regulation of vascular remodeling and angiogenesis. The identification of the specific homeobox genes involved in these processes, their downstream target genes, and the cell signaling pathways activated and repressed by homeobox gene expression in vascular ECs and VSMCs will result in a better understanding of the basic cellular mechanisms by which the vascular system is remodeled in response to physiologic signals, tumors, or other stimuli. Such understanding has the potential to lead to the development of therapies that block tumor angiogenesis and lymphatic metastasis, reverse atherosclerosis, prevent restenosis after angioplasty, improve wound healing, and reverse lymphedema.

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Table I: Homeobox genes expressed in the cardiovascular system

CELL TYPE	GENE	Function/Observation	References
VSMC	*Gax (Mox-2)*	Downregulated upon mitogen stimulation and vascular injury Causes G_1 cell cycle arrest and inhibits VSMC migration Inhibits integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ expression Induces apoptosis in cycling cells Inhibits restenosis after balloon injury Interacts with *Pax3*	(Gorski et al. 1993a, Perlman et al. 1998, Smith et al. 1997, Stamataki et al. 2001, Weir et al. 1995, Witzenbichler et al. 1999, Yamashita et al. 1997)
	Hex	Induces expression of immature actin isoform in VSMCs	(Sekiguchi et al. 2001)
	HOX B7	More highly expressed in fetal VSMCs than adult Induces differentiation of C3H10T1/2 cells into VSMC-like cells	(Bostrom et al. 2000, Miano et al. 1996)
	HOX C9	More highly expressed in fetal VSMCs than adult	(Miano et al. 1996)
	HOX A3 and B3	*HOX A3* knockout mice have vascular anomalies Blocking *HOX A3* and *B3* causes regression of aortic arch 3	(Kirby et al. 1997)
	HOX A5, B5, and C5	Blocking expression causes appearance of additional aortic arch artery	(Kirby et al. 1997)
	HOX A2, A4, A11, B1	Isolated from vascular smooth muscle, functions in VSMC unknown	(Gorski et al. 1993a, Gorski et al. 1994, Patel et al. 1992)
	Prx1	Interacts with SRF to activate binding Putative role in angiotensin II-mediated smooth muscle α-actin expression *Prx1/Prx2* double-null mutants demonstrate vascular anomalies Activates proliferation and tenascin-C expression	(Bergwerff et al. 1998, Bergwerff et al. 2000, Chesterman et al. 2001, Hautmann et al. 1997, Jones et al. 2001)
	Prx2	Widely expressed in embryonic vasculature *Prx1/Prx2* double-null mutants demonstrate vascular anomalies	(Bergwerff et al. 1998, Bergwerff et al. 2000, ten Berge et al. 1998)
Vascular ECs	*HOXA9EC*	EC-specific, function presently unknown Expression modulated by TNF-α	(Patel et al. 1999)
	HOX B cluster	HOX B cluster induced by differentiating factors	(Belotti et al. 1998)
	HOXB3	Involved in regulating capillary morphogenesis	(Myers et al. 2000)
	HOXD3	Induces expression of integrin $\alpha_v\beta_3$ Induces angiogenic phenotype in ECs Impaired function associated with impaired wound healing	(Boudreau et al. 1997, Uyeno et al. 2001)
	HOXD10	Inhibits angiogenesis and changes EC gene expression profile to the nonangiogenic state	(Myers et al. 2002)
	Dlx-3	EST with homology to *Dlx-3* expressed at high levels in tumor endothelium Necessary for placental development	(Quinn et al. 1997, St. Croix et al. 2000)
	Gax (Mox-2)	Inhibits *in vitro* surrogates for angiogenesis May have function in placental-mesenchymal interactions	(Gorski and Leal 2003, Quinn et al. 1997, Quinn et al. 2000)
	Hex	Early marker of endothelial cells during embryogenesis Expressed throughout the vascular network Overexpression increases EC number in embryos Overexpression blocks EC tube formation on Matrigel	(Barbera et al. 2000, Liao et al. 2000, Nakagawa et al. 2003, Newman et al. 1997, Sekiguchi et al. 2001, Thomas et al. 1998)
Lymphatic ECs	*Prox1*	Specific to lymphatic ECs Induces expression of lymphatic EC-specific genes Null mutations prevent development of lymphatic system Master regulator of lymphatic vessel formation from embryonic venous system	(Hong et al. 2002, Petrova et al. 2002, Wigle et al. 1999, Wigle et al. 2002, Wigle and Oliver 1999)

FIGURE LEGENDS

Figure 1. Organization of the HOX clusters. The four Hox clusters in the human and mouse are believed to have evolved through gene duplication. In the human, there are 39 homeobox genes in the HOX clusters (Kosaki et al. 2002). In the mouse, as shown in this figure, the 3' genes are expressed early in embryogenesis in the more rostral regions of the embryo, while the 5' genes are expressed later in embryogenesis in the caudal regions of the embryo (Cillo et al. 1999). The 3' rostral genes are highly responsive to retinoic acid, whereas the 5' caudal genes are less sensitive. Each homeobox gene can have as many as three paralogs in the same position in other HOX clusters. Each HOX cluster is located on a different chromosome. The arrangement of the human HOX clusters, HOX A through D, is nearly identical to the mouse. See text for details.

CURRICULUM VITAE

Personal Information

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Present Position

1999-present	Assistant Professor of Surgery Division of Surgical Oncology UMDNJ-Robert Wood Johnson Medical School The Cancer Institute of New Jersey
2000-present	Member, The Cancer Institute of New Jersey
2000-present	Member, Joint Graduate Program in Cell & Developmental Biology Rutgers University and UMDNJ-Robert Wood Johnson Medical School

Graduate Education

Department and Institution	Degree	Subject	Year Awarded
Department of Physiology and Biophysics Case Western Reserve University Cleveland, Ohio	Ph.D.	Cellular Physiology	1994
University of Michigan Medical School Ann Arbor, Michigan	M.D.	Medicine	1988

Doctoral Dissertation

Homeobox Gene Expression and Regulation in Vascular Myocytes. Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, Ohio. January 1994. Advisor: Kenneth Walsh, Ph.D. (Present location: Department of Cardiovascular Research, St. Elizabeth's Hospital, Tufts University School of Medicine, Boston, MA.)

Undergraduate Education

Department and Institution	Degree	Subject	Year Awarded
Department of Chemistry University of Michigan Ann Arbor, Michigan	B.S.	Chemistry	1984

Postgraduate Training and Experience

Dates	Position
1998-99	Research Associate, Department of Radiation and Cellular Oncology, University of Chicago, Chicago, Illinois.
1988-89	Internship in General Surgery, University Hospitals of Cleveland, Case Western Reserve University School of Medicine, Cleveland, Ohio.
1989-90	PGY-II, General Surgery, University Hospitals of Cleveland, Case Western Reserve University School of Medicine.
1991-93	Flight physician (part time), Metro LifeFlight, MetroHealth Medical Center, Cleveland, Ohio.
1993-96	PGY-III through -V in General Surgery, University Hospitals of Cleveland, Case Western Reserve University School of Medicine, Cleveland, Ohio
1996-98	Fellow, Surgical Oncology, Department of Surgery, University of Chicago, Chicago, Illinois.
1998-99	Research Associate, Department of Radiation and Cellular Oncology, University of Chicago, Chicago, Illinois.

Licensure

Medical Licenses	Expiration Date
State of New Jersey License MA069752	June 30, 2003
State of Ohio License #060908	January 1, 2005

Hospital Appointments

Institution	Position	Dates
Lutheran General Hospital Park Ridge, Illinois	Attending Surgeon, Trauma Services	1996-1999
Robert Wood Johnson University Hospital New Brunswick, NJ	Attending Surgeon, Surgical Oncology and General Surgery	1999 to present
St. Peter's University Hospital New Brunswick, NJ	Attending Surgeon, General Surgery	2000 to present

Certifications

American Board of Surgery certificate number 43113 (expires July 2008).

Professional Organizations/Memberships

American Association for the Advancement of Science	1988-present
American Association for Cancer Research	1999-present
American Society of Breast Surgeons	2000-present

Association for Academic Surgery	2000-present
Society of Surgical Oncology	2002-present
Fellow, American College of Surgeons	2002-present
The Oncology Society of New Jersey	2002-present

Academic Honors, Fellowships, and Awards

Award/Fellowship	Date
Michigan Legislative Merit Award (Scholarship)	1980
Branstrom Freshman Prize	1981
Sophomore Honors Award	1982
Graduation with Honors and High Distinction in Chemistry	1984
Allen Research Fellowship, Department of Surgery, University Hospitals of Cleveland/Case Western Reserve University, Cleveland, Ohio	1990-93
MetroHealth Medical Center Resident Research Award	1994

Committees

Committee	Dates
Admissions Committee Ph.D. Program for M.D.'s Department of Physiology and Biophysics Case Western Reserve University School of Medicine	1992-1993
CINJ Alliance for the Management of Pain (CAMpain) The Cancer Institute of New Jersey UMDNJ-Robert Wood Johnson Medical School and UMDNJ-Robert Wood Johnson University Hospital	2000-present

Patents

Growth Arrest Homeobox Gene Inventors: Kenneth Walsh and David H. Gorski. U. S. Patent no. 5,856,121; issued January 5, 1999.

Extramural funding

Source/Title	Dates	Support/ %Effort
ACTIVE		
DAMD17-02-1-0511 **U.S. Department of Defense Breast Cancer Research Program: Career Development Award** **PI: David H. Gorski**	4/7/2002 – 4/6/2005	50% (Funds salary only)

Source/Title	Dates	Support/ %Effort
Regulation of Breast Cancer-induced Angiogenesis by a Growth Arrest-specific Homeobox Transcription Factor		
DAMD17-03-1-0292 **U.S. Department of Defense Breast Cancer Research Program: Idea Award** **PI: David H. Gorski**	4/14/2003 - 4/13/2006	40% (No salary support)
Inhibition of breast cancer-induced angiogenesis by a diverged homeobox gene		

COMPLETED	Dates	%Effort
Chicago Oral Cancer Center	11/1/1998- 10/31/1999	25%
Angiogenesis Inhibition and Ionizing Radiation in the Treatment of Oropharyngeal Malignancies		
Foundation of UMDNJ (PI: David H. Gorski)	7/1/2000- 6/30/2001	25%
The Role of the Growth Arrest Specific Homeobox Gene Gax in Tumor Cell Biology		
New Jersey Commission on Cancer Research **PI: David H. Gorski**	7/1/2000- 6/30/2002	25%
The Growth Arrest-Specific Homeobox Gene Gax in Cancer		
New Jersey Commission On Cancer Research **PI: David H. Gorski**	7/1/2001- 6/30/2002	25%
The Growth Arrest-Specific Homeobox Gene Gax in Cancer (Supplement for purchase of equipment)		

Publications

Journal articles

1. Merion, R. M., **D. H. Gorski**, G. D. Burtch, J. G. Turcotte, L. M. Colletti, D. A. Campbell (1989). Bile refeeding after liver transplantation and avoidance of intravenous cyclosporine. *Surgery* **106**: 604-610.
2. Patel, C. V., **D. H. Gorski**, D. F. LePage, J. Lincecum, and K. Walsh (1992). Molecular cloning of a homeobox transcription factor from adult aortic smooth muscle. *J. Biol. Chem.* **267**: 26085-26090.
3. **Gorski D. H.**, D. F. LePage, C. V. Patel, N. G. Copeland, N. A. Jenkins, and K. Walsh (1993). Molecular cloning of a diverged homeobox gene that is rapidly down-regulated during the G_0/G_1 transition in vascular smooth muscle cells. *Mol. Cell. Biol.* **13**: 3722-3733.

4. **Gorski, D. H.**, C. V. Patel, and K. Walsh (1993). Homeobox transcription factor regulation in the cardiovascular system. *Trends Cardiovasc. Med.* **3**: 184-190.

5. **Gorski, D. H.**, D. F. LePage, and K. Walsh (1994). Cloning and sequence analysis of homeobox transcription factor cDNAs with an inosine-containing probe. *BioTechniques* **16**: 856-865.

6. **Gorski, D. H.**, and K. Walsh (1995). Mitogen-responsive nuclear factors that mediate growth control signals in vascular myocytes. *Cardiovasc. Res.* **30**: 585-592.

7. Smith, R. C., D. Branellec, **D. H. Gorski**, K. Guo, H. Perlman, J.-F. Dedieu, C. Pastore, A. Mahfoudi, P. Denèfle, J. M. Isner, and K. Walsh (1997). $p21^{CIP1}$-mediated inhibition of cell proliferation by overexpression of the gax homeodomain gene. *Genes Dev.* **11**: 1674-1689.

8. Mauceri, H. J., N. Hanna, M. A. Beckett, **D. H. Gorski**, M. J. Staba, K. A. Stellato, K. Bigelow, R. Heimann, S. Gately, M. Dhanabal, G. A. Soff, V. P. Sukhatme, D. W. Kufe, and R. R. Weichselbaum (1998). Interaction of angiostatin and ionizing radiation in anti-tumour therapy. *Nature* **394**:287-291.

9. **Gorski, D. H.**, H. J. Mauceri, R. M. Salloum, S. Gately, S. Hellman, M. A. Beckett, V. P. Sukhatme, G. Soff, D. W. Kufe, and R. R. Weichselbaum (1998). Potentiation of the anti-tumor effect of ionizing radiation by brief concomitant exposures to angiostatin. *Cancer Res.* **58**:5686-5689.

10. **Gorski, D. H.**, M. A. Beckett, N. T. Jaskowiak, D. P. Calvin, H. J. Mauceri, R. M. Salloum, S. Seetharam, A. Koons, D. M. Hari, D. W. Kufe, and R. R. Weichselbaum (1999). Blockade of the VEGF stress response increases the antitumor effects of ionizing radiation. *Cancer Res.* **59**:3374-3378.

11. **Gorski, D. H.**, and K. Walsh (2000). The role of homeobox genes in vascular remodeling and angiogenesis. *Circ. Res.* **87:** 865-872.

12. **Gorski, D. H.**, H. Mauceri, M. Beckett, R. Salloum, A. Halpern, and R. Weichselbaum (2003). Prolonged treatment with angiostatin reduces metastatic burden during radiation therapy. *Cancer Res.* **63**:308-311.

13. Salloum, R. M., H. J. Mauceri, N. Hanna, **D. H. Gorski**, and R. R. Weichselbaum (2003). Dual induction of the Epo-Egr-TNF-α plasmid in hypoxic human colon adenocarcinoma produces tumor growth delay. *Am. Surg* **69:**24-27.

14. **Gorski, D. H.**, and A. D. Leal (2003). Inhibition of endothelial cell activation by the homeobox gene *Gax*. ***J. Surgical Res.,*** *in press.*

15. **Gorski, D. H.**, A. D. Leal, and J. S. Goydos (2003). Differential expression of vascular endothelial growth factor A (VEGF-A) isoforms at different stages of melanoma progression. ***J. Am. Coll. Surg.,*** *in press.*

16. **Gorski, D. H.**, and K. Walsh (2003). The role of homeobox transcription factors during vascular remodeling and lymphangiogenesis. ***Trends Cardiovasc. Med.,*** *in press.*

17. Goydos, J. S., and **D. H. Gorski.** (2003) Level of expression of vascular endothelial growth factor C (VEGF-C) correlates with stage of local/regional progression in patients with melanoma. ***Clin. Cancer Res.**, in review.*

18. Alsina, J., **D. H. Gorski**, F. J. Germino, W. Shih, S.-E. Lu, Z.-G. Zhang, J.-M. Yang, W. N. Hait, and J. S. Goydos (2003). Detection of mutations in the mitogen-activated protein kinase (MAPK) pathway in human melanoma. ***Clin. Cancer Res.**, in review.*

19. **Gorski, D. H.** (2003). Regulation and function of the growth arrest-specific homeobox gene *Gax* in vascular endothelial cells during angiogenesis *in vitro* and *in vivo. In preparation.*

20. Salloum, R. M., H. J. Mauceri, M. Beckett, N. Hanna, **D. H. Gorski**, and R. R. Weichselbaum (2003). Gene therapy using the dual induction of TNF-α by hypoxia and ionizing radiation results in an enhanced antitumor effect. *In preparation.*

Published abstracts

1. **Gorski, D. H.** (2001) Negative regulation of angiogenesis by a growth arrest-specific homeobox gene. *J. Surg. Res.* **100**: 288-289.

2. **Gorski, D. H.** (2002) The homeobox gene *Gax* induces p21 expression and inhibits vascular endothelial cell activation. *Ann. Surg. Oncol.* 9:S42.

3. **Gorski, D. H.** and J. Goydos (2002). Differential expression of vascular endothelial growth factor (VEGF) isoforms at different stages of melanoma progression. *J. Am. Coll. Surg.* 195:S63.

Editorials

1. **Gorski, D. H.**, and K. Walsh (2001). Control of vascular cell differentiation by homeobox transcription factors. *Circ. Res.* **88**:7-8.

Book chapters

1. **Gorski, D. H.**, H. Mauceri, and R. Weichselbaum. Strategies for combining gene therapy with ionizing radiation to improve antitumor efficacy. *Gene Therapy of Cancer: Translational Approaches from Preclinical Studies to Clinical Implementation*, Second Edition. Lattime, E.C., and S. L. Gerson, eds. Academic Press, San Diego (2002).

Abstracts

1. *Radiation and angiostatin synergistically inhibit the growth of Lewis lung carcinoma.* **D. H. Gorski**, H. Mauceri, N. Hanna, S. Gately, G. Soff, and R. Weichselbaum. The Society of Surgical Oncology Meeting, San Diego, California, March 27-29, 1998.

2. *The anti-tumor effect of angiostatin is potentiated by brief concomitant exposure to angiostatin.* **D. H. Gorski**, H. Mauceri, S. Gately, G. Soff, R. Weichselbaum. The Society of Surgical Oncology Meeting, Orlando, Florida, March 4-7, 1999.

3. *Angiostatin and ionizing radiation suppress lung metastases in Lewis Lung carcinoma.* **D. H. Gorski**, H. Mauceri, R. R. Weichselbaum. The Society of Surgical Oncology Meeting, New Orleans, LA, March 16-19, 2000).

4. *Improved tumor response of esophageal adenocarcinoma to combined treatment with anti-VEGF antibody and radiation.* N. Jaskowiak, **D. Gorski**, R. Salloum, H. Mauceri, S. Seetharam, R. Weichselbaum, M. Posner. The Society of Surgical Oncology Meeting, New Orleans, LA, March 16-19, 2000).

5. *A new isocoumarin, NM3, selectively inhibits endothelial cell growth* in vitro. R. Salloum, H. Mauceri, N. Jaskowiak, **D. Gorski**, S. Seetharam, D. Hari, M. Posner, D. Kufe, R. Weichselbaum. The Society of Surgical Oncology Meeting, New Orleans, LA, March 16-19, 2000.

6. *The growth arrest-specific homeobox gene* Gax*: A potential negative regulator of angiogenesis.* **D. Gorski**. The Society of Surgical Oncology Meeting, Washington, D.C., March 15-18, 2001.

7. **Gax***: A growth arrest-specific homeobox transcription factor that inhibits VEGF-induced proliferation and tube formation in endothelial cells.* **D. Gorski**. American Association for Cancer Research Meeting, New Orleans, LA, March 24-28, 2001.

8. *The homeobox gene* **Gax** *induces p21 expression and inhibits vascular endothelial cell activation.* **D. Gorski**. The Society of Surgical Oncology Meeting, Denver, CO, March 14-17, 2002.

9. *The growth arrest-specific homeobox gene* **Gax** *blocks cell cycle progression and migration and induces p21 expression in vascular endothelial cells.* **D. Gorski** and A. Leal. American Association for Cancer Research Meeting, San Francisco, CA, April 6-10, 2002.

10. *The level of vascular endothelial growth factor C (VEGF-C) expression correlates with lymphatic density and stage of progression in patients with melanoma.* **D. Gorski** and J. Goydos. American Association for Cancer Research Meeting, Toronto, Ontario, Canada, April 5-9, 2003.

Non-print Publications

Genbank DNA sequences

Sequence Designation	**Database/ Accession No.**	**Description and year**
RATHOX111A	Genbank M91802	*R. norvegicus* homeobox protein (*HOXA2*) mRNA, complete sequence. (1992)
RATHOX1III	Genbank L03556	*R. norvegicus HOXA5* mRNA, 3' end. (1992)
RATHOX1IV	Genbank L03557	*R. norvegicus* HOXA4 protein mRNA, 3' end. (1992)
RATZNFI.	Genbank L03386	*R. norvegicus* zinc finger protein mRNA, 3' end. (1992)
RNGAXMR	EMBL Z17223	*R. norvegicus* mRNA encoding Gax protein. (1993)

Scientific Presentations

1. *Gax: A novel homeobox gene that causes cell-cycle arrest in vascular myocytes.* Resident Research Competition, MetroHealth Medical Center/Case Western Reserve University School of Medicine. May 1994.
2. *Synergism between angiogenesis inhibition and radiation therapy in control of Lewis lung carcinoma growth.* Charles Huggins Research Conference, The University of Chicago, Chicago, Illinois. May 2, 1998.
3. *The anti-tumor effect of angiostatin is potentiated by brief concomitant exposure to angiostatin.* **D. H. Gorski**, H. Mauceri, S. Gately, G. Soff, R. Weichselbaum. Plenary Session, The Society of Surgical Oncology Meeting, Orlando, FL, March 4-7, 1999.
4. *The growth arrest-specific homeobox gene* Gax*: A potential negative regulator of angiogenesis.* D. Gorski. Cancer Forum, The Society of Surgical Oncology Meeting, Washington, D.C., March 15-18, 2001.
5. *Negative regulation of angiogenesis by a growth arrest-specific homeobox gene.* D. Gorski. Association for Academic Surgery Meeting, Milwaukee, WI, November 15-17, 2001. *J. Surg. Res.* **100**: 288-289 (2001).
6. *Differential expression of vascular endothelial growth factor (VEGF) isoforms at different stages of melanoma progression.* D. Gorski. Surgical Forum, American College of Surgeons Meeting, San Francisco, CA, October 7, 2002.

Presentations and Lectures

1. *Homeobox genes in vascular smooth muscle cells: Toward a gene therapy of blood vessel diseases?* Department of Surgery Grand Rounds, Case Western Reserve University, Cleveland, Ohio. March 19, 1994.
2. *Oncogenes and tumor suppressors: A review with potential clinical applications.* Department of Surgery Grand Rounds, Case Western Reserve University, Cleveland, Ohio. March 5, 1995.
3. *Optimal surgical treatment of malignant melanoma.* Surgical Oncology Grand Rounds, University of Chicago, Chicago, Illinois. May 28, 1997.
4. *Esophageal cancer: resection versus nonsurgical treatment.* Surgical Oncology Grand Rounds, University of Chicago, Chicago, Illinois. October 29, 1997
5. *Angiostatin and ionizing radiation target the tumor vasculature.* Dartmouth Medical School, Dartmouth-Hitchcock Medical Center, Lebanon, New Hampshire. February 4, 1998.
6. *Total mesorectal excision in the management of rectal cancer.* Surgical Oncology Grand Rounds, University of Chicago, Chicago, Illinois. February 18, 1998.
7. *Treatment of hilar cholangiocarcinoma.* Surgical Oncology Grand Rounds, University of Chicago, Chicago, Illinois. June 3, 1998.
8. *Targeting tumor radiotherapy to tumor vasculature using anti-angiogenic therapy.* Greenebaum Cancer Center, University of Maryland, Baltimore, MD; November 13, 1998.

9. *Anti-angiogenic therapy as a means of targeting radiation therapy to the tumor vasculature.* Medical University of South Carolina, Charleston, SC; December 16, 1998.

10. *Antiangiogenic radiotherapy.* Lombardi Cancer Center, Georgetown University, Washington, D.C.; January 7, 1999.

11. *Antiangiogenic therapy targets radiotherapy to tumor vasculature.* The Cancer Institute of New Jersey, University of Medicine and Dentistry at New Jersey/Robert Wood Johnson Medical School, New Brunswick, NJ; February 12, 1999.

12. *Angiogenesis.* The Cancer Institute of New Jersey Annual Retreat, Princeton Marriott, Princeton, NJ, May 10, 2000.

13. *Angiogenesis.* Passaic General Hospital, Mountainside Hospital Nursing Education Program, November 17, 2000.

14. *Negative regulation of angiogenesis by the homeobox gene* Gax. Presented at the University of California at San Francisco, Department of Surgery, Surgical Research Laboratory, San Francisco General Hospital, April 9, 2002.

15. *Angiogenesis.* Grand Rounds. Department of Surgery, UMDNJ-Robert Wood Johnson Medical School, Robert Wood Johnson University Hospital, New Brunswick, NJ, February 12, 2003.

16. *Angiogenesis.* The Cancer Institute of New Jersey, UMDNJ-Robert Wood Johnson Medical School, New Brunswick, NJ. April 4, 2003.

Editorial Activity (manuscript review)

Blood
Cancer Research
Clinical Cancer Research

Grant Review

Reviewer for Comitato Telethon Fondazione ONLUS (Telethon Italy)